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COLOURATION AS A SIGNAL OF HEALTH STATUS.

The effects of diet, parasites and androgens on the expression of colour in the European Serin (*Serinus serinus*)

Tese de Doutoramento em Biologia, Especialidade de Ecologia, orientada pelo Professor Doutor Paulo Gama Mota e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Título: A coloração como sinalizador do estado de saúde. Avaliação do efeito da dieta, parasitas e androgénios na expressão da coloração na Milheirinha (*Serinus serinus*).

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Fotografia de um macho de Milheirinha *Serinus serinus*

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SUMMARY

In this study, yellow carotenoid-based plumage colouration of a cardueline finch, European serin *Serinus serinus*, was studied by laboratory manipulations and field work. Carotenoid-based plumage colouration is widespread in birds and a sexually selected trait in many passerines, as in this model species. The aim of this study was to investigate the factors that affected the production of the yellow patch in males, namely diet, hormones, parasites and immunocompetence, combining laboratory manipulations and field work. I further explored the function of the trait in females.

With a diet laboratory manipulation, modifying the intake of a non-pigmentary carotenoid during moult, I found that plumage colours could be a signal of general condition and antioxidant status. Carotenoid supplemented males had higher levels of plasma carotenoids and higher immune response to an immune challenge. Moreover, supplemented males were colourful and selected in a mate choice experiment. This way, high quality males were colourful males, possibly giving direct and indirect advantages to females.

If colourful males are selected by females, what was colouration signalling? I wanted to explore the predictors of plumage colouration in a field study, with free wild birds. In a four years study, in the beginning of the breeding season, I took morphometric and colorimetric measurements. The statistical models revealed that colouration could be predicted by age and ectoparasite load. Then I made a comparison between two different colorimetric approaches, the human oriented tristimulus colour variables and the avian visual models, based on the physiology of avian eyes. I found that these two colorimetric approaches were highly correlated.

Extrinsic factors, as diet and parasites, had a great effect on colouration, and intrinsic factors, as age, could also affect the expression of the trait. There are other mechanisms that could control secondary sexual traits, and in males, androgens, are assumed to have a role on it. It was expected that testosterone, the main male hormone, had an effect on this trait. In order to test this prediction, I performed a testosterone manipulation on

males' serins during moult and found that testosterone had a limited and negative effect on the expression of the carotenoid-based yellow patch.

Finally, as females also present some variation on the colouration of the breast, I wanted to explore this signal in females. The signal could be used in inter or intra-sexual contexts, and I wanted to test both. Therefore, I performed male mate choice trials and competition between females over food. Contrary to my expectations, I found that female colouration in this species is not sexually selected neither used in intra-sexual competition. I concluded that, in females, this trait could be result of genetic correlation of the males' trait.

Keyword: European serin; *Serinus serinus*; colouration; plumage; carotenoids; diet; testosterone; mate choice; parasites.

RESUMO

Neste trabalho estudou-se, através de manipulações laboratoriais e trabalho de campo, a coloração da plumagem baseada em carotenóides de um tentilhão carduelino, a Milheirinha *Serinus serinus*. A coloração baseada em carotenóides está bem distribuída em aves e é um traço seleccionado sexualmente em passeriformes, como é o caso da nossa espécie modelo. O objectivo deste estudo foi o de investigar os factores que afectam a produção do sinal amarelo em machos, nomeadamente a dieta, hormonas, parasitas e imunocompetência, combinando manipulações laboratoriais e trabalho de campo. A mensagem do sinal nas fêmeas também foi explorada.

Com uma manipulação laboratorial da dieta, alterando a ingestão de um carotenóide não pigmentário durante a muda, descobri que a cor da plumagem pode ser um sinal da condição geral e da capacidade antioxidante do indivíduo. Os machos com o suplemento de carotenóides tinham níveis de carotenóides no plasma e respostas a desafios imunitários mais altos. Além disso, os machos com o suplemento de carotenóides eram mais coloridos e foram seleccionados numa experiência de selecção de par. Deste modo, indivíduos de alta qualidade eram mais coloridos, provavelmente fornecendo vantagens directas e indirectas às fêmeas.

Se os machos mais coloridos são seleccionados pelas fêmeas, o que está a coloração a sinalizar? Foram explorados os preditores da coloração da plumagem em aves silvestres, num estudo de campo ao longo de quatro anos. No início da época de reprodução, foram recolhidas medidas morfométricas e de coloração. Os modelos estatísticos revelaram que a coloração pode ser induzida pela idade e carga de ectoparasitas dos indivíduos. Posteriormente foi feita uma comparação entre os dois modos de analisar a coloração, as variáveis *tristimulus* baseadas na visão humana e os modelos visuais de aves, baseados na fisiologia dos olhos de aves. Descobri que as duas formas de colorimetria estão altamente correlacionadas.

Factores extrínsecos, como dieta e parasitas, tiveram um grande efeito na coloração, e factores intrínsecos, como a idade, podem também afectar a expressão do traço. Existem outros mecanismos que podem controlar os traços sexuais secundários. Em

machos, assume-se que os androgénios são um desses mecanismos, e é previsível que a testosterona, a principal hormona masculina, tenha um efeito sobre esta característica. De modo a testar esta hipótese, realizei uma manipulação dos níveis de testosterona no sangue dos machos durante a muda e descobri que a testosterona tem um efeito limitado e negativo sobre a expressão da mancha amarela com base em carotenóides.

Finalmente, como as fêmeas também apresentam alguma variação da coloração do peito, eu queria explorar este sinal nas fêmeas. O sinal pode ser usado em contextos inter- ou intrasexuais, e eu queria testar ambos. Deste modo, realizei experiências de selecção de par dos machos e competição por alimento entre fêmeas. Ao contrário do esperado, a coloração feminina nesta espécie não é sexualmente seleccionada nem usada em competição intrasexual. Concluí que, nas fêmeas, essa característica pode ser resultado da correlação genética da característica dos machos.

Palavras-chave: Milheirinha; *Serinus serinus;* coloração; plumagem; carotenóides; dieta; testosterona; escolha de par; parasitas.

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This thesis originated four articles, two submitted and under revision and two in preparation. Chapters 2, 3 and 4 were executed and written by me, with the exception of hormone assay in chapter 4, which was performed by an independent laboratory from ISPA. Field and laboratory assistance was provided by Ana V. Leitão and Filipe Rocha. Chapter 5 was done in collaboration with Ana V. Leitão. I am responsible for the experiment in intersexual selection and Ana V. Leitão for intrasexual competition experiments. Professor Paulo Gama Mota provided guidance for planning and structure all the experimental procedures and preparation of manuscripts.

Chapter 2 originated a paper in second revision in Behavioural Ecology and Sociobiology:

Trigo S., Mota P. G. What is the value of a yellow patch? Assessing the signalling role of yellow colouration in the European serin.

Chapter 4 originated a paper in second revision in Ecological Research:

Trigo S., Mota P. G. A test of the effect of testosterone on a sexually selected carotenoid trait in a cardueline finch.

Chapter 3 correspond to a paper under preparation for submission to Functional Ecology:

Trigo S., Mota P. G. Age and parasites predict carotenoid-based plumage colour on male European serin.

Chapter 5 correspond to a paper under preparation for submission to Animal Behaviour:

Trigo S., Leitão A. V., Mota P. G. What is females' carotenoid-based plumage colouration signalling?

CHAPTER 1

General Introduction

The focus of this thesis was on the evolution, mechanisms, functions and information content of one of the most conspicuous secondary sexual traits of Birds: the plumage colouration. I address these questions using as a model a cardueline finch, the European Serin, *Serinus serinus*, in laboratory tests, with dietary and hormonal manipulations, mate choice trials and analysis of field data. In this initial chapter, I present a theoretical framework and knowledge about sexual selected signals, on how they evolved, what information they provide and how they are formed is presented. The following chapters are organized in the form of complete independent papers. Finally, I present the main conclusions in a concluding chapter.

1. SEXUAL AND SOCIAL SELECTION

The presence of conspicuous ornaments, apparently detrimental to the survival of animals, posed a problem to the theory of evolution by natural selection, developed by Charles Darwin. To overcome this, Darwin proposed a new concept, sexual selection as being "the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction" (Darwin 1871). Sexual selection occurs when individuals differ in their reproductive success and this difference could occur between individuals of the same sex that compete for access to mates, and in this case is designated as intra-sexual selection; or it can occur between individuals of different sexes, when one sex exerts a mating choice over members of the opposite sex, and in that case is called inter-sexual selection. These two selective processes are non-mutually exclusive, and can even be reinforced (Berglund et al. 1996).

Usually, females are the choosy sex due to fundamental differences between males and females, namely, gamete size and differences in parental care (Kokko and Jennions 2003). There is now abundant evidence that larger males, with bigger weapons or more colourful ornaments achieve the highest mating success. By choosing, females may

acquire some direct benefits, like increased fecundity, parental care or nuptial gifts (Trivers 1972) or acquire indirect benefits or good genes (Johnstone 1995). According to the good genes model, secondary sexual characters are likely to be condition dependent in their expression (Jennions et al. 2001).

The evolution of female preferences could be explained by several mechanisms, probably with some acting simultaneously (Andersson 1994). Individuals could receive direct benefits from their mates, as increased fecundity or increased number of offspring. This mechanism of evolution is common and well supported in empirical studies (Møller 1994). Female preference for mates could also be a by-product of natural selection on sensory systems. This sensory bias model of sexual selection assumes that natural selection is the predominant evolutionary mechanism that affects preference (Fuller et al. 2005). Ornament preference should evolve when they honestly signal genetic advantage or direct benefits for females. Besides, female preference and male ornamentation might evolve together, a process known as runaway sexual selection, with several genetic evidences for this mechanism (Mead and Arnold 2004). The selected traits could also be indicator signals, signalling overall good condition of individuals, with associated costs. Only high-quality individuals could afford to maintain the signal. There are several mechanisms explaining the honesty of sexual signals, one of the most accepted is the handicap model proposed by Zahavi (1975). Zahavi's handicap model states that the honesty of a signal is measured by its costs, which can be physiological, energetic, social or other. In order to be evolutionary stable, a signal must honestly signal the trait and there must have a cost (Grafen 1990). Hamilton and Zuk (1982) proposed that sexual ornaments are indicators of parasite and disease resistance. Parasites could affect the development of ornamental traits, and females prefer males who are not infected, supporting the idea that parasites are an important factor in sexual selection. Besides, females may choose genetically compatible mates, in a way to obtain optimal fit offspring.

2. CAROTENOID-BASED PLUMAGE COLOURATION AS A HONEST SIGNAL

Physiological costs and limitations could constrain the evolution of colouration, and in this way colouration could honesty signal individual quality. Bright plumage colouration of birds is an example of evolution of traits by sexual selection and frequently signals physical condition, health or parasite resistance (Andersson 1994; McGraw and Ardia 2003). In particular, carotenoid colouration of male passerine birds is thought to be used by female mate choice (Hill 2006b) and sexual selection should be stronger for carotenoid-based rather than melanin-based colouration (Badyaev and Hill 2000). Carotenoid-based plumage colouration is a complex trait, which could be signalling multiple quality aspects and could be affected by different individual parameters.

Carotenoids are responsible for the red, orange and yellow colouration of sexual ornaments (McGraw 2006) and are a common trait preferred by females (Peters 2007). Carotenoids cannot be synthetized by vertebrates, and can only be acquired in their diet (McGraw 2006), which can limit their expression on plumage. Besides acting as colorants, carotenoids have a variety of physiological roles in birds. One of the recognised functions of carotenoids is at the level of cells protection from oxidative damage (Burton 1989) and boosting of the immune system (Chew 1993; McGraw and Ardia 2003). However, at very high concentrations, carotenoids can have a pro-oxidant action (Young and Lowe 2001).

Identifying the limit factors in the pathway between nutritional access and colouration is essential to understand how carotenoid-based ornaments evolved and are maintained as honest signals (McGraw 2006). Complex interactions between carotenoids, testosterone, parasites and immune capacity could be crucial to the role of honesty in carotenoid-based signals (McGraw and Ardia 2003), which have not been tested yet (Blas et al. 2006).

2.1. Carotenoids

Carotenoids are molecules that can be divided in two classes accordingly to the presence or absence of oxygen. Carotenes (as β-carotene) are non-oxygenised carotenoids, non-polar and lipid soluble, whereas xanthophylls (as zeaxanthin, lutein) contain oxygen and are much more polar (McGraw 2006) (Figure 1).

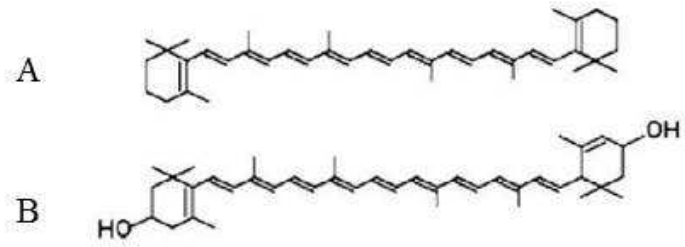

Figure 1 Chemical structure of carotenoids common in birds' diets: (A) β-carotene and (B) lutein.

Vertebrates can obtain carotenoids by consuming algae, fungi and plants or by ingesting animal preys rich in carotenoids. After the ingestion, carotenoids must be absorbed. In vertebrates, carotenoids diffuse through the intestine along with some lipids into bloodstream. They can after be modified into different forms (Brush 1990) (Figure 2). The main dietary carotenoids in birds are lutein, zeaxanthin, the carotenes (α and β), and the cryptoxanthins (α and β) (McGraw 2006). Carotenoids could be present in birds' serum in a very variable range of concentrations (Tella et al. 2004) and plasma carotenoid concentration is related to integumentary (McGraw and Gregory 2004; Negro et al. 1998) and plumage colouration (Hill et al. 1994). In feathers, carotenoid pigments can protect from UV radiation (Bortolotti 2006), have a role on thermoregulation and protect from bacterial degradation (Grande et al. 2004).

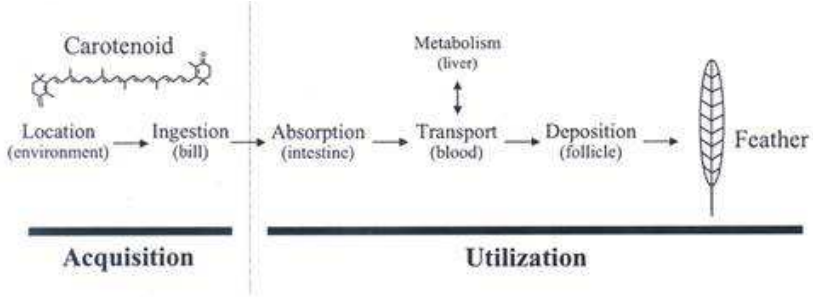

Figure 2 Pathway of carotenoids from acquisition through deposition in the integument of the birds. In parentheses is the site in which each stage occurs. Adapted from McGraw and Hill 2001.

Yellow to red carotenoid-based plumage also reflect in the UV part of the spectrum. Recent research has shown that in feathers all colours are produced by an interaction of pigments and feather structure (Fitzpatrick 1998; Prum 2006; Shawkey et al. 2006; Shawkey and Hill 2005). The typical reflectance curve of serins yellow plumage, with canary xanthophyll A and B (Stradi et al. 1995, 1996), is represented in Figure 3, with one peak in the UV part of the spectrum and a plateau starting proximally at 550 nm.

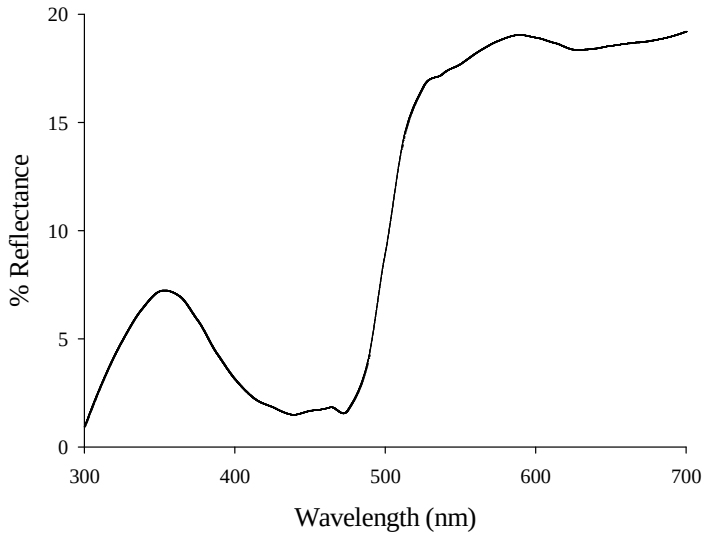

Figure 3 Reflectance spectrum from the yellow carotenoid-based plumage of European serins *Serinus serinus*. Raw data from the breast of a male.

2.2. Hormonal control of colouration

Hormones regulate several behavioural, physiological and morphological processes in animals, and are thought to mediate trade-offs because they produce antagonistic effects on different body components (Ketterson and Nolan 1992). Colouration of plumage and bare parts in birds are under hormonal and nonhormonal regulation (Kimball 2006). The hormonal regulation of colouration includes oestrogens, androgens and the peptide hormone, luteinizing hormone (LH) (Kimball 2006). Testosterone, a steroid androgen hormone produced by the testes, is the main male hormone (Peters 2007) and its effects are well known on several life processes, including reproduction and development of secondary sexual traits. Testosterone has also been associated with physiological and behavioural costs on individuals (Wingfield et al. 2001). These costs can be increased basic metabolic rate (Buchanan et al. 2001), increased level of stress hormones (Ketterson and Nolan 1992) or decreased immunity (Casto et al. 2001; Mougeot et al. 2004; Verhulst et al. 1999). Thus, testosterone could provide a mechanism for reinforcing the honesty of ornament signals.

Two hypotheses have been formulated to explain testosterone effect on honesty signal reinforcement mechanism. The immunosuppression of testosterone leads to the formulation of the immunocompetence handicap hypothesis (ICHH), suggesting that ornaments and immune system compete for resources (Folstad and Karter 1992). Only individuals with good immune systems could withdraw the costs of high testosterone levels and develop elaborate ornaments. More recently, the oxidation handicap hypothesis (OHH) suggests that high testosterone levels induce oxidative stress, and the ornament signals the trade-off between expression and resistance to oxidative stress (Alonso-Alvarez et al. 2007; Alonso-Alvarez et al. 2008).

In birds, testosterone is involved in development of song, sexual ornaments and behaviour (Alatalo et al. 1996; Ball et al. 2003; Ketterson and Nolan 1992; Wingfield et al. 2001; Zuk et al. 1995). Testosterone controls bird colouration based on melanin (Bókony et al. 2008). It increases the bib size of house sparrows *Passer domesticus* (Buchanan et al. 2001; Evans et al. 2000; Gonzalez et al. 2001) and is involved in the

development of nuptial plumage in males superb fairy-wrens *Malurus cyaneus* (Peters et al. 2000). Carotenoids used in ornaments can also be modulated by testosterone (Andersson 1994; McGraw et al. 2006), but to date the influence of testosterone has been seen mainly in bare parts and the skin. The few studies that investigate the relationship between carotenoid-based plumage colouration and testosterone found contradictory results. In blue tits *Cyanistes caeruleus*, testosterone enhances structural carotenoid colouration (Roberts et al. 2009); but in red-legged partridges *Alectoris rufa* the presence of an effect was dependent on age (Alonso-Alvarez et al. 2009).

2.3. Parasites and colouration

Birds could have a variety of parasites with differently ways of affecting ornamentation. Hamilton and Zuk (1982) proposed that secondary sexual characters evolved because they honestly signal resistance to parasites and disease. Accordingly to this hypothesis most carotenoid-based colouration should be sensitive to parasite infection (Lozano 1994) and indeed, parasites have been shown to negatively affect expression of carotenoid-based ornamentation (Baeta et al. 2008; Mougeot et al. 2007).

Coccidia (phylum Apicomplexa, sub-order Eimeriorina) are protozoan parasites, whose oocysts are present in the faeces of animals. These infections are very common in wild birds, and in most birds, coccidian cause non-fatal chronic infections. When coccidia encyst in the gut line, cause a thickening of the epithelium, which inhibits the absorption and transport of carotenoids (Hill 2006a). Negative relationships between coccidian infection and carotenoid-based colouration were found in different passerine species; house finches (Brawner et al. 2000), American goldfinches *Carduelis tristis* (McGraw and Hill 2000) and greenfinches *Carduelis chloris* (Hõrak et al. 2004).

Ectoparasites have also been proposed to affect carotenoid colour expression. Feather mites abundance during moult negatively affects the plumage colouration in a series of species: house finches *Carpodacus mexicanus* (Thompson et al. 1997), wren

Troglodytes troglodytes, dunnock *Prunella modularis*, robin *Erithacus rubecula*, blue tit, great tit *P. major*, chaffinch *Fringilla coelebs*, greenfinch, linnet *C. cannabina*, and yellowhammer *Emberiza citronella* (Harper 1999). In serins, the abundance of feather mites during moult was negatively correlated with the plumage colouration developed (Figuerola et al. 2003). Also, feather-degrading bacteria could change the appearance of colouration, presumably by affecting structural colour (Shawkey et al. 2007). Although results are not clear, with some authors finding no relation (Cristol et al. 2005) and others finding positive relations (Shawkey et al. 2007).

Despite these results and due to the lack of knowledge about the real host–parasites interactions, some authors indicate that there is no evidence of all of these parasites having a negative effect on host condition and fitness. Colouration could reflect the overall intensity of parasite infection rather than the individual parasite load or absolute parasite burden (Biard et al. 2010).

2.4. Immunocompetence and colouration

The immune system of vertebrates comprises three levels of defence, acting altogether. The first is physical barriers that protect the entry of infectious agents into the body, like skin and mucous secretions (Delves et al. 2009). The second level is provided by the innate immune system, a generalist and effective system. Although being highly efficient, the innate immune responses are not always enough to cope with infectious agents. So, the immune system has one last level of defence, the specific acquired immune system, i.e. the adaptive elements. The acquired immune responses take a few days to be active but they are specific to an infectious agent. This response is mediated primarily by T- and B-lymphocytes, that recognize the antigen (Delves et al. 2009).

The complexity of the immune system could only be assessed by an array of assays, and the majority of studies of immunocompetence in behavioural ecology have used

only one or two immunological assays (Garamszegi et al. 2004; Hõrak et al. 2006; Roberts and Peters 2009; Saks et al. 2006). We choose to use two of the mostly used immune challenges in bird studies: the phytohaemagglutinin-P (PHA-P) and sheep red blood cells (SRBC).

The PHA-P injection assay is used to evaluate the proliferation of multiple immune cells and involves both the innate and adaptive elements of the immune system (Martin et al. 2006). The relationship between this immune response and colouration is not clear, with positive (McGraw and Ardia 2003; Zuk and Johnsen 2000), negative (Karadas et al. 2005) and null results (Biard et al. 2009; Navara and Hill 2003; Saks et al. 2003; Svobodová et al. 2013).

The SRBC challenge mimics a challenge to an individual's immune system by a novel pathogen and, in that way, controls for prior exposure, acquired immunity or differences in susceptibility, and involves the adaptive immune system. SRBC immunization triggers T- and B-cell responses, including initial antigen recognition and presentation and production of specific antibodies (Ros et al. 2008), and for that reason SRBC immune challenge is considered to be an estimate of the acquired immune system. SRBC responses positively relate with ornamentation in some species (McGraw and Ardia 2003; Saks et al. 2003), but have no relation on others (Navara and Hill 2003).

2.5. Female colouration and sexual selection

Traditionally, the elaboration of ornamental traits has been generally described focusing on males while the evolution of female colouration has been neglected (Amundsen 2000). Females have a higher parental investment, for gametes or after fertilization, so receptive females are rarer (Kokko and Jennions 2003). Males have a higher potential reproduction rate, due to the production of small low cost gametes and to a more reduced parental investment. Also, variation in operational reproductive ratio

may render receptive reproductive females scarcer than reproductive males so that, typically, males compete for access to females. As females have a lower potential reproductive rate, mate choice became much important, and females carefully choose a mate in order to get the best survival descendent (Kokko and Jennions 2003). However, conspicuous female colouration is widespread among birds (Clutton-Brock 2009; Kraaijeveld et al. 2007) and there are three main explanations for female colouration: the genetic correlation (Kraaijeveld et al. 2007; Lande 1980), the mutual mate choice (Clutton-Brock 2007; Johnstone et al. 1996) and social selection (West-Eberhard 1983).

The genetic correlation hypothesis explains female colouration as non-functional by-products of sexual selection over male traits (Kraaijeveld et al. 2007; Lande 1980). The mutual mate choice hypothesis states that although males are normally the most competitive sex, females could be similarly competitive and be chosen (Johnstone et al. 1996). This should occur when males make a large contribution to parental investment. And the social selection hypothesis explains that female colouration could have evolved due to interactions between individuals outside the context of reproduction, involving sexual and nonsexual competitions (Lyon and Montgomerie 2012). Social competition could be over some ecological resources, like food, shelter or nesting material (West-Eberhard 1983) and influences the evolution of weapons, ornaments and behaviour in both males and females.

2.6. Status signalling

There is some evidence that avian ornamental colour of plumage and bare parts also have a role in status signalling, besides sexual signalling (Kraaijeveld et al. 2004). In conflict situations, males try to evaluate fighting ability of opponents, avoiding direct confrontation (Maynard Smith and Harper 1988). A large body of empirical support for status signalling came from studies with passerines. Plumage colours could be used to assess individual fighting ability, establishing dominance in contests and acting as a

badge of status. Plumage status signalling is often associated with melanin (Senar 2006), nevertheless, carotenoid-based status signals are present in different bird species, as yellow warblers *Dendroica petechia* (Studd and Robertson 1985), red-collared widowbirds *Euplectes ardens* (Pryke et al. 2001), red-shouldered widowbirds *E. axillaris* (Pryke and Andersson 2003) and rock sparrow *Petronia petronia* (Griggio et al. 2007).

3. MEASURING PLUMAGE COLOURATION

Different animals perceive colours in different ways. The eyes of most vertebrates have several cone photoreceptors cells that are sensitive to different parts of the spectrum. In the photoreceptor cells there are coloured oil droplets that function as light filters, changing the sensitivity of those cones and thus, changing colour vision (Vorobyev 2003). Birds perceive colours in a different way than humans, due to three main differences between human and avian colour vision (Figure 4). The first is that birds have a broader spectral range than humans; the lower limit of the spectrum is about 400 nm for humans and about 300 nm for birds (Cuthill et al. 2000; Hart 2001). The second difference between humans and birds is in the number of cone types in the retina, humans have three and birds had four colour cone types (Maier and Bowmaker 1993). Birds have long (LWS), medium (MWS), short (SWS) and ultraviolet (UVS) or violet (VS) waves (Cuthill 2006) possessing tetrachromatic vision. And the last difference between avian and human vision is the oil droplets that filter the light entering the cones (Cuthill 2006) and increases colour discrimination (Vorobyev, 2003) (Figure 4). Due to these differences, specific technical methods are needed to access avian colouration considering their sensorial capacities. Nowadays, the most common method to quantitatively measure bird colouration is to determine a reflectance

spectrum in the 300 nm to 700 nm using a portable spectrophotometer and then to calculate some variables from those data (Montgomerie 2006).

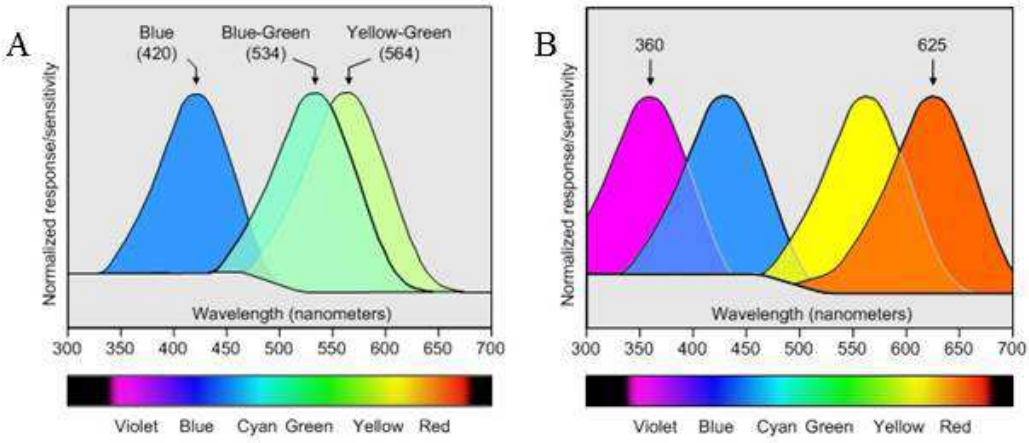

Figure 4 Spectral sensitivities of visual pigments in the single cones of humans (A) and a bird (B) (adapted from www.diycalculator.com).

One of the most common colour descriptions used in the study of birds has been tristimulus variables: hue, saturation and brightness (HSB) (Table 1), that correspond to the three major axes of colour variation perceived by humans. Hue represents "colour", saturation is the "purity" of colour and brightness is the "intensity", an index that can be used to compare individuals and species (Montgomerie 2006).

Table 1 Tristimulus colour variables (Montgomerie 2006) used in the analyses of birds' colours. $\boldsymbol{R_{\lambda i}}$ represents the percentage of reflectance at $\boldsymbol{\lambda_i}$ (wavelength) and $\boldsymbol{n_w}$ the number of wavelengths intervals used.

Colour variable	Formula
Hue (H)	$H = \lambda_{Rmid} = (R_{max} + R_{min})/2$
Saturation (S)	$S = (R_{max} - R_{min})/B$
Brightness (B)	$B = \sum_{320}^{700} R_i / n_w$

The popularity of the HSB tristimulus model is based on the easy human visualization and interpretation. However these variables do not represent exactly the birds' ability to perceive colours.

Just recently, researchers have started to investigate avian signals from the avian perspective, with some authors' recommendation to use instead avian visual models, based on the birds' physiology and perception (Butler et al. 2011). These models are the only ones that closely assess what birds perceive; an estimate of photon catch of each birds' single cone receptor is calculated, based on the irradiance spectra of incident light, the reflectance properties of feathers, the transmission properties of air and the birds' ocular media and the spectral sensitivities of the birds' retinal cones (Vorobyev et al. 1998). Since the data for all species is it not known, commonly is used a close species as estimate. There are several approaches to these models, but the most commonly used is the tetrachromatic visual model by Vorobyev and colleagues (Vorobyev and Osorio 1998; Vorobyev et al. 1998). This type of visual model describes colour by chromatic and brightness variables, taking into account feather reflectance, ambient illumination, and background colour.

4. MODEL SPECIES

European serins are small cardueline finches, socially monogamous and gregarious. Males sing virtually all year round, although more intensively during reproductive season, stimulating nest-building behaviour (Mota 1999; Mota and Depraz 2004). Male European serins exhibit bright yellow colouration produced by carotenoids (Stradi et al. 1995) on forehead, supracilium, throat, breast, and uropygium. On the back they present green-brownish colour with grey-brownish strikes. This species is sexually dimorphic, with females being drabber than males (Figure 5). Juveniles are more similar to females,

and moult their plumage into adult plumage during the autumn of the first year (Cramp and Perrins 1994).

European male serins use canary xanthophylls A and B as major carotenoids in feather colouration (Stradi et al. 1995), which are synthesised at feather follicle from lutein and zeaxanthin acquired from diet (McGraw and Gregory 2004). The serin diet is almost composed by Brassica seeds, with some occasional small invertebrates (Cramp and Perrins 1994).

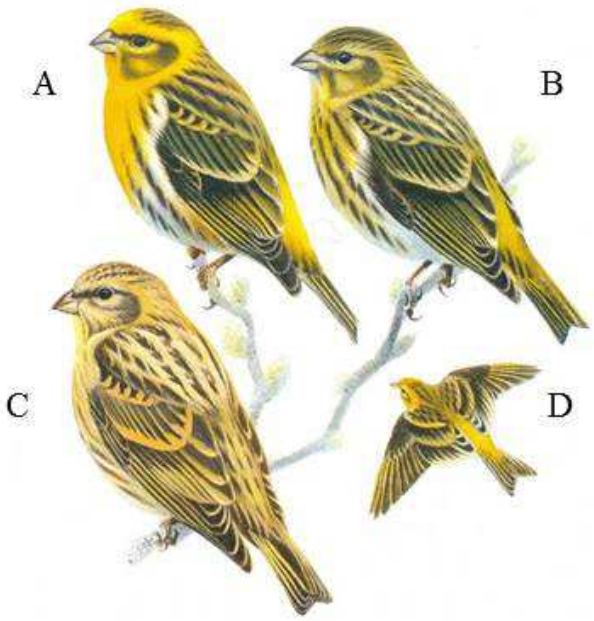

Figure 5 Representation of European serins (*Serinus serinus*). A: adult male; B: adult female; C: Juvenile; D: adult male in flight. Adapted from Clement et al. 1993.

The reproductive season takes place between late February and July, with males singing intensively during the entire breeding season. This species is socially monogamous with semi-colonial breeding grounds (Mota and Hoi-Leitner 2003). During breeding season, male serins guard their mates and perform intense extra pair behaviour, however with low (Hoi-Leitner et al. 1999) or no extrapair paternity detected (Mota and Hoi-Leitner 2003). In the serins the parental care is high and shared by males

and females (Mota and Hoi-Leitner 2003). In this species, vocal communication is vital and high vocal interaction is present between individuals (Mota 1999; Mota and Depraz 2004).

5. OBJECTIVES AND OUTLINE OF THE THESIS

With this research I wanted to investigate the mechanisms and factors acting on the expression of the European serin plumage colouration, and determine if this trait is an honest signal. I wanted to study if there is a relationship between sexual ornamentation with physical condition, parasite load, immunocompetence, hormones, and diet.

High quality individuals should be able to forage and metabolise food better, enhancing their ornamentation. Therefore, diet manipulation of males allowed me to test if carotenoid-based ornamentation is nutrition dependent. In order to determine what were females choosing, males were measured in the beginning of the reproductive season and a relationship between colouration, age, physical condition, and parasites was investigated. Another objective was to test the hypothesis of testosterone-dependent carotenoid-based ornaments, with more colourful males being able to cope with higher testosterone levels. Moreover, I wanted to disclose if female plumage colouration could function as a signal, used in intra or inter sexual selection, or could be only a genetic correlative trait.

In more detail, my objectives were:

1. Experimentally modify the access to carotenoids and investigate its effect on immune response, plasma carotenoid levels and the expression of plumage colouration;
2. Quantify the relationship between morphometric and physiological variables and colouration expression;

3. Experimentally modify the plasma androgen levels and investigate the effects on physical condition and the expression of plumage colouration;
4. Investigate the function of plumage colouration of females.

5.1. Chapter 2: Nutritional control of the signal

In chapter 2, I experimentally modified the birds' diet and investigated the effects on blood carotenoid concentration, immune responses, and physical condition. Moreover, it was observed the effect on plumage colouration and female preference.

An honest signal should be costly to produce, and sexual selection favours the evolution of honest signals, as carotenoid-based colouration. These pigments have several physiological functions, besides acting as colorants, so a trade-off for carotenoid availability has been suggested. Recently, it has been proposed that carotenoid colouration could signal the overall anti-oxidant quality of an individual (Hartley and Kennedy 2004). The antioxidant machinery is modulated by vitamins, namely vitamin A; animals can't synthetize vitamin A, but could obtain it from diet or metabolize from vitamin A precursors, such as β-carotene (Hill and Johnson 2012). I wanted to evaluate if the availably of dietary β-carotene could affect the condition, colouration expression and female preference in this species. Through a long experiment, I found that β-carotene effectively enhanced immune responses, plumage colour and female preference, supporting the possibility of an indirect role of diet in yellow carotenoid colouration.

5.2. Chapter 3: Colouration, age, body condition and parasites

In chapter 3 I wanted to study if there was a link between colouration, age, parasite load, and morphometric variables in male serins, through a field work with free living

birds. Due to the condition-dependence of carotenoid-based colours, it is expected that older, high-quality males are more colourful.

In a four year period, during pair formation and breeding season, I captured and measured male serins in the wild. I found that colour plumage expression could be predicted by age and ectoparasite load. I also evaluated two different colorimetric techniques, tristimulus variables and models of avian colour vision, and found that they were highly correlated.

5.3. Chapter 4: Hormones and signal expression

In this chapter I experimentally manipulated the hormone concentration of males during moult in order to access the effects on physical condition and plumage colouration expression.

Androgens regulate several physiological functions of individuals and many male secondary traits, as well as reproductive behaviour (Mougeot et al. 2003). Carotenoid-based colouration is a common sexual trait in birds and could also be modulated by testosterone (Blas et al. 2006; Peters 2007). I implanted serin males with testosterone before plumage moult and evaluated the effects on plumage colouration and physical condition, during and after moult. I found that testosterone levels during moult had only a negative effect on the size of the yellow plumage patch, revealing a limited effect of testosterone on a carotenoid-based plumage colouration. Although not significantly different between groups, testosterone treatment negatively affected UV-chroma and nearly saturation. Besides, contrary to expected, testosterone did not decrease males' physical condition.

5.4. Chapter 5: Female signal colouration

In chapter 5, I wanted to disclose if the colouration of the plumage of females could be a signal used in sexual or social contexts or a non-functional by-product of selection on male ornaments.

In this species, females also present plumage colouration, although they are drabber than males. I wanted to test if this ornament has a sexual function or a social function by performing respectively, a male mate choice trials and a test of social competition for access to limited food. I also studied a possible link between plumage colouration and physical condition, age and parasites, and found none. With the male mate choice trials I found no evidence for sexual selection on carotenoid-based ornamentation. Males prefer females that were available, independent of their colouration. Further, in social competition tests, although females formed steep hierarchies, dominance was not associated with ornamentation. These joint results suggest that in this species, the variation on female plumage colouration is a result of genetic correlation with the males' trait.

5.5. Chapter 6: Conclusions

In this chapter I briefly discuss the main results from all the previous chapters. I present general conclusions and possible research lines for future studies.

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CHAPTER 2

What is the value of a yellow patch? Assessing the signalling role of yellow colouration in the European serin

ABSTRACT

Sexual selection promotes the evolution of signals many of which can reliably indicate condition, health, or good genes of individuals. In order to be evolutionarily stable, indicator signals must be costly to produce. Carotenoid colouration evolved in many species by sexual selection. Carotenoids besides acting as pigments have been implicated in immune defence and anti-oxidation which makes them likely candidates for honest signalling. A trade-off for carotenoid availability was proposed as the basis for signal honesty. Alternatively it was suggested that carotenoid colouration is not advertising the presence of the pigment *per se*, but the quality of anti-oxidant resources which then affect carotenoid concentration. One possibility is that carotenoid-based colouration could signal colourless antioxidant mechanisms, which are partially regulated by vitamins. β-carotene is one of the most common precursors of vitamin A, and although present in birds diet, is not available for feather colouration. If an indirect association exists between carotenoid signal and condition then manipulation of β-carotene concentration could reveal that this link is indirect.

We tested this by conditioning the availability of β-carotene in the diet of a cardueline finch with yellow carotenoid colouration. β-carotene-supplemented males had higher plasma carotenoid concentration and higher response to a cellular immunity challenge (PHA) than control males. β-carotene-supplemented males also had more saturated plumage colouration and were preferred by females in a mate choice test. Our results support the possibility of an indirect role for yellow carotenoid colouration.

Keywords: carotenoid-based ornamentation; immune response; colouration; sexual signals; sexual selection.

INTRODUCTION

Sexual selection is an evolutionary process that favours the evolution of a class of signals which are indicators of quality (Andersson 1994). The evolution of these signals is dependent on the existence of costs for their production and maintenance, since only signals that are costly can be 'honest' indicators of quality (Zahavi 1975; Grafen 1990; Searcy and Nowicki 2005). Carotenoid colouration is widespread among vertebrates and is frequently involved in sexual signalling (Olson and Owens 1998; Møller et al. 2000), including most of the yellow, orange and red colouration of the integuments of birds, reptiles and fishes (Olson and Owens 1998) and is widely accepted to be condition dependent, linked to individual ability to acquire, assimilate and process carotenoids (Hill 1990; Hill 1999). The conspicuous plumage colouration of birds is a main example of signal evolution by sexual selection (McGraw 2006). Carotenoid colouration is one of the most widespread type of social signals in birds and, one of the best studied kind of ornamental traits, being involved in sexual communication, nestling signalling and mate choice (Hõrak and Saks 2003). For example, it was shown that females prefer to mate with males that display more intense carotenoid colouration in house finches *Carpodacus mexicanus* (Hill 1990; Hill 1994; Hill et al. 1999; Toomey and McGraw 2012), American goldfinches *Carduelis tristis* (Johnson et al. 1993), yellowhammers *Emberiza citronella* (Sundberg 1995), zebra finches *Taeniopygia guttata* (Simons and Verhulst 2011) and serins *Serinus serinus* (Leitão et al. 2014).

Carotenoids act as pigmentary molecules of bright plumage, fleshy tissues and other bare parts, but they can also be stored in the liver, or fat depots from which they might be mobilised (Ninni et al. 2004). According to the pigment allocation hypothesis (Lozano 1994), the maintenance of honesty of sexual signals is assured by two non-mutually exclusive factors: 1) dietary carotenoids are a limiting resource (Endler 1983) and 2) carotenoids have antioxidant functions and modulate immune responses. A trade-off was assumed to exist between the use of carotenoids in ornamental colouration and in several physiological functions, which would assure the honesty of the signal (Blount 2004). These pigments are not synthesized by vertebrates, so they have to be acquired

through their diet as intact macromolecules (Goodwin 1984; McGraw 2006), which can be a limiting factor through pigment availability, and can contribute to the signal reliability of the animal's foraging capacity and condition. Other costs could contribute to the honesty of the signal such as those related to maintaining physical condition or to other fitness related traits (Lozano 1994; McGraw and Ardia 2003; Blas et al. 2006; Pérez-Rodriguez et al. 2010). It is known that these pigments are health-related, enhancing immune function and antioxidant activity (Lozano 1994; Olson and Owens 1998; Blount et al. 2003; Faivre et al. 2003; reviewed in Blount 2004). This was recently supported in a meta-analysis by Simons et al. (2012). Carotenoids are thought to be responsible for enhancing immunity mediated by cells, antibody production, gene expression, and for enabling protection to cells and tissues from oxidation (Chew and Park 2004). They can also inhibit mutagenesis and have a role in photoprotection (Bendich and Olson 1989; Krinsky 1989).

There is clear evidence that carotenoid availability affects colour expression and immune response. In several species of birds, carotenoid-supplemented males had higher immune responses than non-supplemented males (Fenoglio et al. 2002; McGraw and Ardia 2003; McGraw and Ardia 2005; Aguilera and Amat 2007), and improved growth and survival (Saino et al. 2003; Biard et al. 2006). Also, more colourful birds had higher immune responses (Saks et al. 2003; Mougeot 2008) and experienced less oxidative stress (Pérez-Rodriguez et al. 2010). Conversely, immune activation caused a decrease in colouration and plasma carotenoid levels (Alonso-Alvarez et al. 2004; Peters et al. 2004; Baeta et al. 2008; Pérez-Rodriguez et al. 2008). However, other studies have failed to find a relationship between carotenoids and oxidative stress *in vivo* (El-Agamey et al. 2004; Hartley and Kennedy 2004). In addition, in high dosages, carotenoids could even have a pro-oxidant activity (Bertrand et al. 2006a; Costantini and Möller 2008; Huggins et al. 2010).

Countering the trade-off hypothesis for the honesty of carotenoid signalling Hartley and Kennedy (2004) suggested that carotenoids might not signal directly the carotenoid antioxidant capacity, but instead signal the quality of other antioxidant resources of the

animal. Antioxidants are molecules that scavenge free radicals, thus preventing oxidative stress to damage cells (Surai 2002; Martínez et al. 2008), and these antioxidant molecules include vitamins C, E and A, and antioxidant enzymes (Hartley and Kennedy 2004). Vitamin A has a variety of functions on basic life processes such as vision, reproduction, growth and development, and also on redox homeostasis, and is obtained from animal tissues or derived from β-carotene and other pro-vitamin A carotenoids (Biesalski et al. 2007). Thus, if the availability of carotenoids which do not take part in colouration was increased, and there was both an increase in health related functions and in colouration, then this would constitute a proof for the indirect signalling role of carotenoid colouration. β-carotene is a powerful antioxidant molecule (Bendich 1989; Krinsky 1989; Chew 1993), possesses immunoregulatory activities (Bendich 1989; Chew 1993; Cucco et al. 2006) and it is one of the most important vitamin A precursors (Chew 1993), and also is not involved in feather colouration. If β-carotene can protect pigmentary carotenoids from oxidation, it is possible that it also affects carotenoid uptake into feather colouration, and signal expression in an indirect way.

In order to test this we conducted a full year-round study, manipulating β-carotene availability for male European serins (*Serinus serinus*) during moult, testing its effect on immune and physical condition, on plumage expression, and lastly testing its effect on female choice over these males in the following breeding season. The European serin is a small social sexually dichromatic seed-eater finch (Cramp and Perrins 1994) with males exhibiting a carotenoid-based yellow plumage (Stradi et al. 1995a) which goes through a single post reproductive moult (Pagani-Nuñez and Senar 2012). The colouration of serin feathers is the result of deposition of canary xanthophylls A and B (Stradi et al. 1995b), resulting from oxidization of dietary lutein (McGraw et al. 2001). Carotenoid colouration has been shown to be sexually selected in serins (Leitão et al. 2014) and related to survival in the wild (Pagani-Nuñez and Senar 2012). Thus we predict that: 1) β-carotene-supplementation will enhance the plasma carotenoid levels, immune system and physical condition of males, 2) β-carotene-supplementation will

enhance the colouration of males and 3) β-carotene-supplemented males are preferred by females in mate choice experiments.

METHODS

Subjects and housing

Males were captured in the winter (months 1 - 2 in Fig. 1), with mist nets in agricultural fields nearby Coimbra, Portugal. Birds were ringed and housed at the Department of Life Sciences, University Coimbra until the end of the experiments (month 17 in Fig. 1), in wired cages, under natural light and ventilation, with *ad libitum* access to a commercial food mixture (European Finches Prestige, Versele-Laga, composition: canary seed 46%, rapeseed 22%, niger seed 7%, linseed 7%, peeled oats 6%, hempseed 5%, wild seeds 5%, radish seed 1% and spinach seed 1%), tap water and commercial mix grit with oyster shell. All males had *ad libitum* access to the same seed mixture and had a supplement of glucose two times a week. These conditions allowed males to moult on a natural light regime. A subset of those birds was subject to mate choice tests in the spring of 2010.

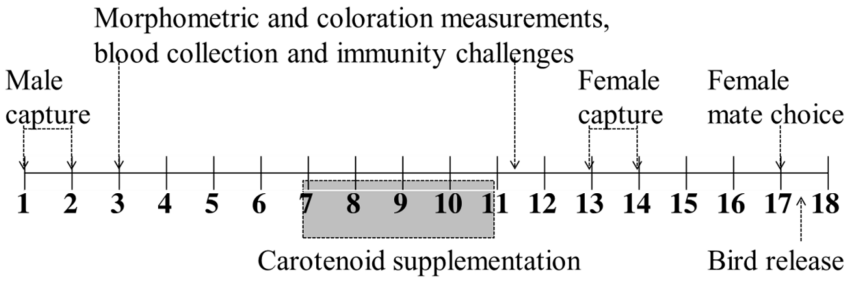

Figure 1 Experimental timeline. Numbers are months (Month 1 = January 2009).

Morphometric and colouration measurements, blood collection and immunity challenges were made before and after the carotenoid supplementation (months 3 and 11 in Fig. 1).

Physical condition was calculated as the residuals of a regression of body mass over tarsus length, a reliable and the most used estimate of condition (Jakob et al. 1996; Ots et al. 1998). The relationship between the two variables was linear, with residuals over tarsus having an even distribution (Schulte-Hostedde et al. 2005). Ectoparasite mite load on wing feathers was assessed by an estimating method following Behnke et al. (1995; 1999).

Carotenoid supplementation

Males were randomly assigned to two treatment conditions before moult: β-carotene-supplementation (β-supplemented-males) that received daily 0.2 g/l β-carotene diluted in water as a substitute for water, and control non-supplemented males (control), which only received water. This carotenoid dosage was estimated by comparison with other studies (e.g. Navara and Hill 2003) and by a previous experiment in our laboratory. The carotenoid supplementation lasted for the entire moult period (months 7 to 11 in Fig. 1).

Measurement of carotenoid plasma concentration

Plasma carotenoid concentration was determined by transmission spectrophotometry, following a protocol that provides good estimates of plasma total carotenoid concentration, which are highly correlated with results from high-performance liquid chromatography (Alonso-Alvarez et al. 2004; Aguilera and Amat 2007). Carotenoids were quantified by diluting the plasma into 100% acetone (1:10), vortexed for 5 s and centrifuged, at 1000 rpm for 10 min, to precipitate the flocculent protein. The

absorbance of the supernatant was measured with a transmission spectrophotometer Shimadsu UV-1601, at 476 nm. The total plasma carotenoids concentration (µg/ml) was calculated using a standard curve of Lutein 'α-carotene-3,3'-diol' (Sigma-Aldrich).

Tests of immunity response

Two immunity challenges were performed: the Sheep Red Blood Cells (SRBC) haemagglutination assay and the Phytohaemagglutinin (PHA-P) wing web assay. SRBC antigens challenges T-dependent humoral immunity (Ots et al. 2001; Hasselquist and Nilsson 2012) and PHA-P wing web challenges the immunity mediated by cells, involving both innate and adaptive responses of the immune system (Martin et al. 2006; Tella et al. 2008). For the SRBC assay, males were inoculated, intra-abdominally, with 20 µl of 2% SRBC in PBS (phosphate buffered saline). A week later about 100 µl of blood was collected from birds, centrifuged, and the plasma was preserved in –20ºC. Plasma was used to perform a haemagglutination assay using a base 2 serial dilution. The trite of the antibody was given by the last well with agglutination. For the PHA-P wing web test we used a protocol following Smits et al. (1999) by measuring the wing web of males twice (with values being averaged) before inoculation. Birds were then injected in the wing web with a suspension of 20 µg PHA-P (Sigma-Aldrich L-8754, USA) in 20 µl PBS, and the wing was measured again after 24 h, following the same procedure. The intensity of response was assessed through wing swallowing between the two measurement days. We used a calliper to the nearest 0.01 mm to measure wing web thickness at the injection point. All measurements were made by the same researcher (PGM), who was unaware of birds' treatment condition.

Colouration measurements

We measured the plumage colouration of males with a spectrophotometer Ocean Optics USB4000 (Ocean Optics, Dunedin, FL, USA), with deuterium and halogen light source (Mikropack Mini-DT-2-GS, UV-VIS-NIR), emitting light between 300 nm and 700 nm, and an optical fibre reflectance probe (Ocean Optics R400-7 UV/VIS), held vertically, attached to a rigid black holder to standardise the distance between probe and sample (3 mm), providing a sampling area of 28 mm^2. All measurements of the spectrum were expressed as the proportion of light relative to a white standard (Ocean Optics, WS-1-SS White Standard). We took measurements in four different areas: forehead, throat, breast and belly, making three readings from each sampled area which was averaged. For each area, we calculated tristimulus colour variables from spectral reflectance data between 320 and 700 nm, including the UV region (320 to 415 nm), to which birds are sensitive (Cuthill 2006): brightness, UV-brightness, saturation, UV-chroma and hue (Montgomerie 2006). Brightness was computed as $\sum_{\lambda 320}^{\lambda 700} R_i/n_w$ (1), UV-brightness as $\sum_{\lambda 320}^{\lambda 415} R_i/n_w$ (2), saturation was computed as $(R_{max} - R_{min})/brightness$ (3), UV-chroma as $(R_{maxUV} - R_{min})/brightness$ (4) and Hue as $\lambda R_{mid} = (R_{max} + R_{min})/2$ (5); where $R_{\lambda i}$ is the percentage of reflectance at λ_i (wavelength), n_w is the number of wavelengths intervals used (Montgomerie 2006). For data reduction, we performed three Principal Component Analysis (PCA) for saturation and UV-chroma (hereafter designated as saturation), for brightness and UV-brightness (hereafter designated as brightness) and for hue, before and after treatment. 1st factor of PCA for saturation explained 46.7% of variation, before treatment, and 39.1% of variation, after treatment. For brightness, before treatment, the 1st factor of PCA explained 39.9% of variation and, after treatment, 53.3% of variation. Finally, for hue, before treatment, the 1st factor explained 53.9% and, after treatment, 58.8% of variation. All variables had positive loads on the 1st factor. The area of the yellow patches of forehead and chest were measured by overlapping transparent grids and counting the number of squares covering these areas.

Mate choice experiment

Females were captured during the winter (months 13 - 14 in Fig. 1) and were housed in separate cages in the same facilities, but with no visual contact with males. The mate choice experiments were performed in a test room, with three compartments (main: 155 x 272 x 220 cm; smaller: 112 x 136 x 220 cm) (details in Leitão et al. 2014). This two-way apparatus has the best performance in this kind of test, with low estimation errors (Bruzzone and Corley 2011). The aviary apparatus had full-spectrum fluorescent lights (Philips TL950 Full Spectrum Fluorescent). During trials, a female was placed in the main compartment, facing the two males and separated from them by a glass. The two males were in adjacent compartments separated by an opaque wall. Twelve females were used in the tests performed in early spring (Month 17 in Fig. 1). Each female was tested only once against a pair of males, one from each treatment group. Males were randomly assigned to each of the two compartments in order to eliminate possible female positional preferences. No combination of two males was repeated. The trials lasted 45 min, being the first 15 min considered habituation time. Tests were video-recorded and the analysis was performed with the Observer 5 software (Noldus, Wageningen, The Netherlands). The closest area to each male's compartment in the female's compartment was designated as female's "choice area" (see Fig. 1 in Leitão et al. 2014). We used time spent by females in the interaction area of males as a measurement of female preference (Nolan and Hill 2004 and wherein references).

Statistical analysis

We performed a one-way ANOVA before and after the diet experiment, to test for differences between the two groups. The female mate choice tests were analysed through a Generalised Linear Model (GLMs) with repeated measures (with normal error distribution), having individual female as subject and the female time spent in the choice area as dependent variable and male type as within subject factor for pairwise

comparisons. To control for male behaviour we included male treatment group and time spent by male in the female interaction area as fixed effects. The Wald χ^2 statistic was used to test for significance in the GLMs. Sample sizes was not equal for all measured variables, due to problems with blood collection or insufficient plasma volume. So we report sample sizes in all analysis. All statistical analyses were performed with IBM SPSS Statistics 19.0.

RESULTS

There was no initial difference in colour expression between the two groups of males (Table 1), as well as in plasma carotenoid levels ($F_{1,11} = 1.097$, $P = 0.320$) and physical condition ($F_{1,17} = 0.599$, $P = 0.450$). Also, there were no differences in the responses to the two immunity challenges: SRBC ($F_{1,10} = 2.157$, $P = 0.176$) and PHA-P ($F_{1,11} = 2.847$, $P = 0.122$), and in ectoparasite load ($F_{1,17} = 0.119$, $P = 0.734$).

Table 1 Differences between control and β-supplemented-males for colouration variables, before and after supplementation. N = 18.

	Before treatment		After treatment	
	F	P	F	P
Saturation	0.001	0.955	4.869	**0.042**
Brightness	0.003	0.872	0.546	0.471
Hue	0.027	0.872	1.392	0.255
Forehead patch	1.928	0.184	0.411	0.532
Chest patch	0.172	0.684	0.000	0.996

Carotenoid plasma level, immune responses and physical condition

The β-carotene-supplementation experiment successfully created a difference in carotenoid levels, as β-supplemented-males had higher levels of plasma carotenoid concentration than control males, after the treatment (control males: 1.20 ± 0.446 µg/ml; β-supplemented-males: 7.22 ± 2.192 µg/ml; F1,11 = 7.253, P = 0.023). β-supplemented-males showed a higher response in the PHA-P immune challenge than control males (F1,11 = 8.949, P = 0.014) (Fig. 2A), but there were no differences in the SRBC immune test (F1,10 = 0.567, P = 0.469) (Fig. 2B).

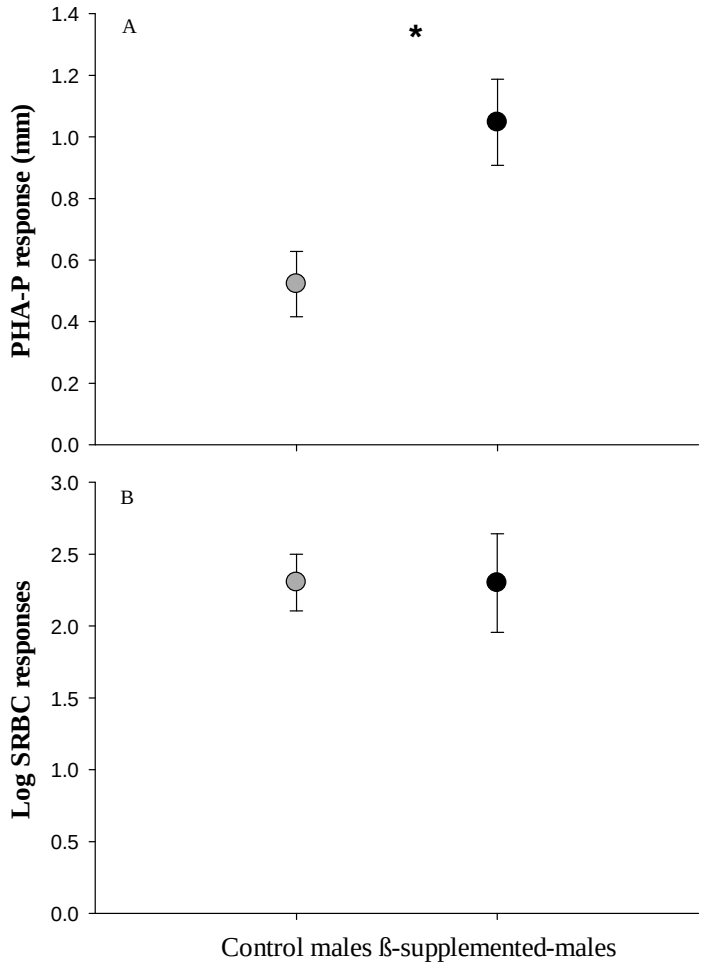

Figure 2 Control and β-supplemented-males responses to the two immune challenges: PHA-P and SRBC. (A) males response to PHA-P ($F_{1, 11}$ = 8.949, p = 0.014); B) males response to SRBC ($F_{1, 10}$ = 0.567, p = 0.469). Results are presented as mean ± standard error.

After the treatment, the two groups of males presented no differences in ectoparasites load ($F_{1,11} = 1.000$, $P = 0.341$) or in physical condition ($F_{1,11} = 1.856$, $P = 0.203$).

Carotenoid-based colouration

After the food-supplementation experiment, the colouration of β-supplemented-males was more saturated than that of the control males (Fig. 3) (Table 1). There were no differences in brightness and hue, or in the size of the colouration patch both in the forehead and the chest (Table 1).

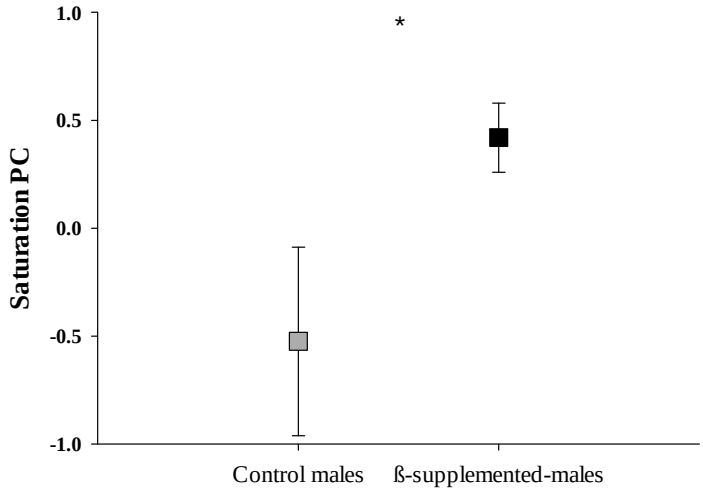

Figure 3 Plumage saturation for control and β-supplemented-males after moult. Values of saturation are given by the first factor of a principal component analysis for saturation. Results are presented as mean ± standard error. * Indicates significant differences ($p < 0.05$, $N = 18$)

Mate choice experiment

The two treatment-groups of males were submitted to a female choice test in the following breeding season in order to assess the effect of colour change in mate choice. Females clearly preferred β-supplemented-males, spending significantly more time facing them (Wald χ^2 = 5.434; d.f. = 1; p = 0.02) (Fig. 4) than control males. This was not affected by the time males spent in front of the female (Wald χ^2 = 0.102; d.f. = 1; p = 0.750).

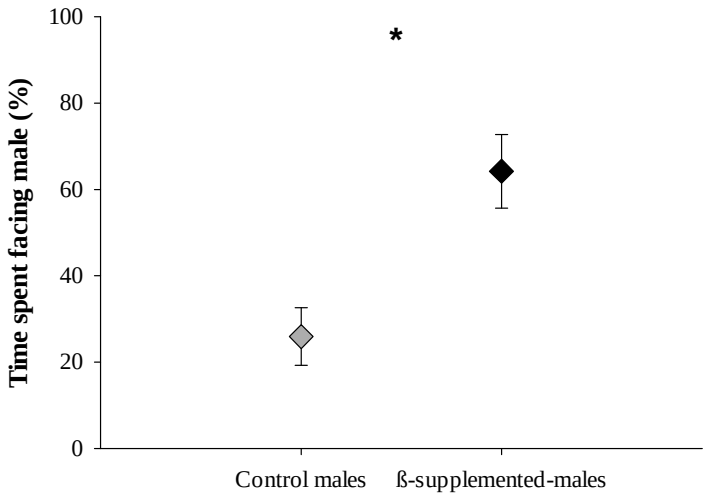

Figure 4 Time spent by females in association with β-supplemented-males and control males (N = 12), in the following spring

DISCUSSION

Our experiments revealed that by increasing β-carotene availability in the diet of male serins we also observed an increase in their carotenoid plasma concentration and in their immune response. Our treatment also affected plumage ornament expression and attractiveness to females, since males given extra β-carotene became more colourful and were preferred by females over control males. Our three initial predictions were

confirmed, which supports and indirect role for carotenoid colouration as signal of condition. Serins undergo a single post-reproductive moult, which takes place long before the signal is relevant for sexual display. So, carotenoid-based plumage colouration in this species should, most likely, predict long-term aspects of individual quality.

Carotenoid plasma level, immune responses and physical condition

First we wanted to determine if the addition of β-carotene to the diet of males affected their immune response. Indeed, β-supplemented-males showed an increase in plasma carotenoid concentration and a stronger immune response. Other experiments showed that an enhanced diet promoted a higher plasma lutein circulation on great tits *Parus major* (Peters et al. 2011) and a nutritional deprivation diminished plasma carotenoids in male goldfinches (McGraw et al. 2005). β-carotene is a carotenoid particularly linked to an antioxidant role and is an immunoenhancer (Bendich 1989; Chew1993), and it also serves as vitamin A precursor, which is involved in several basic metabolic processes, including growth, development, vision, immune system and reproduction (D'Ambrosio et al. 2011).

Our results revealed that the β-carotene-supplementation affected particularly the cellular immunity since the immune response of males was only significant in the PHA-P test, which measures the immunity mediated by cells, involving both innate and adaptive responses of the immune system (Martin et al. 2006; Tella et al. 2008). No differences were found between supplemented and control males in the SRBC test, which measures T-dependent humoral immunity. In male red grouse *Lagopus lagopus scoticus*, comb colour and condition predicted the PHA-P response (Mougeot 2008), and in red-legged partridges, *Alectoris rufa* colouration, plasma carotenoids and cell-mediated immune response were positively correlated (Pérez-Rodriguez et al. 2008). Also, in a diet experiment during moult of great tits carotenoid supplementation increased PHA-P response, but not SRBC response (Peters et al. 2011). And in a recent

meta-analysis, Simons et al. (2012) found that PHA was the only measure of immune function that was associated with carotenoid levels. The immune challenge response seems to be species-specific however, as contrasting results can be found in different species. Navara and Hill (2003) reported no differences in immune responses to increasing doses of carotenoid supplementation in American goldfinches. But, in another study, carotenoid supplementations increased both cell-mediated and humoral immune responses in zebra finches (McGraw and Ardia 2003). While in some other studies β-carotene was responsible for the increase in antibody titres in cockerels (McWhinney and Bailey 1989) and wild gulls (Blount et al. 2001). One possible mechanism for the action of β-carotene on immune stimulation could be through mitochondrial function on the immune system. Mitochondria have well known roles in cellular metabolism, generating energy for physiological processes, regulating stress responses and signalling for apoptotic cell death (West et al. 2011; Galluzzi et al. 2012). Besides, mitochondria could have a central role in the innate immunity (West et al. 2011). As several mitochondrial functions are regulated by vitamin A (Stillwell and Nahmias 1983), an indirect action of β-carotene and vitamin A on immune system could occur.

Although there was an increase in immune response of the β-supplemented-male serins, we found no differences in physical condition or in ectoparasite load between these and control males. Ectoparasite load was actually very similar between the two treatment groups, which is probably due to moulting occurring in the nearly aseptic aviary environment.

Carotenoid-based colouration as a signal

We also wanted to determine if supplementation with β-carotene could affect the yellow colour expression of males, which could only occur through an indirect effect, as

β-carotene is not in the metabolic pathway to produce the pigments to be deposited in the birds' feathers (McGraw 2006).

Under natural conditions, birds have a limited access to carotenoids due to environmental constrains or experience. A critical assumption for the evolution of sexually selected signals is that they have to be honest about the traits they signal, which depends on them being costly (Grafen 1990; Searcy and Nowiki 2005). A trade-off between using carotenoids for immune defence and for colour signalling was suggested as being the main cause for the maintenance of the honesty of the signal (Blount 2004). Similarly, carotenoid colouration was associated with antioxidant function (Chew and Park 2004). However, this trade-off hypothesis was questioned in the sense that, most likely, carotenoids are not environmentally limiting. Instead, it was proposed that carotenoid colouration was not advertising their direct antioxidant function but was acting as an indirect indicator of uncoloured resources including antioxidant molecules which could protect carotenoids from oxidation (Hartley and Kennedy 2004). In accordance with this hypothesis Bertrand et al. (2006b) found an additive effect of carotenoids and melatonin, which is a free radical scavenger in bill colour in zebra finches. If the concentration of carotenoids in feathers is not directly related to their availability for this and other purposes, but is instead a signal of general health of individuals, then improving the individuals' condition, e.g. by making other carotenoids available, should also affect the expression of the signal. By supplementing male serins with β-carotene we assessed the indirect effects that high concentration of carotenoid may have as antioxidants, immune enhancers, or as acting in other metabolic processes which ultimately affect the transformation of lutein and zeaxanthin into canary xanthophylls A and B that are mobilized into feathers. Our results agree with carotenoid colouration being an indirect indicator of condition-associated resources which can also be carotenoid dependent and susceptible of improvement by carotenoid availability in serins.

More recently a vitamin A-redox hypothesis was proposed by Hill and Johnson (2012) linking carotenoid colouration and individual oxidative state and immune function, through the cellular pathways that are regulated by vitamin A, which is an

essential micronutrient and plays a major role in several basic life processes, as redox homeostasis. The hypothesis was advanced to explain the signalling role of red colouration in birds, since carotenoids can act as vitamin A precursors and also be deposited in feathers after modification. In yellow coloured birds the process is different since the main carotenoids in feathers, canary xanthophylls A and B, are obtained from oxidation of lutein and zeaxanthin, while pro-vitamin A carotenoids follow a different pathway, and are either transported to the liver or cleaved into retinal (Debier and Larondelle 2005; von Lintig 2010). The authors consider that their hypothesis can also apply to species with yellow colouration, albeit in a different way, since vitamin A not only regulates carotenoid uptake and transport, but also acts as antioxidant maintaining redox levels. Although we do not know by which mechanism β-carotene is affecting these birds colouration, our results are in accordance with this hypothesis, since the increase of the main vitamin A precursor availability had an effect in the colour saturation of males. The vitamin A-redox hypothesis establishes a detailed set of possible connections between carotenoid colouration and the biochemical and molecular processes of vitamin A homeostasis and oxidative state. One possibility to explain our results is that carotenoid ornamentation signals individual oxidative state, which is highly mediated by vitamin A; hereby the ingestion of β-carotene could affect the trait. Another possibility is that besides its role as vitamin A precursor, β-carotene could have a role on the immune system or on individual homeostasis, thus affecting immune responses and plumage colouration. Finer testing on the mechanisms that relate condition with signal expression in species with carotenoid colouration is needed. There are only a few examples linking non-pigmentary substances and colouration of a carotenoid-based sexual trait. Beak colour was associated with carotenoid and vitamin A concentration in spotless starlings (*Sturnus unicolor*) (Navarro et al. 2010) and a non-pigmentary antioxidant enhanced bill colour in zebra finches (Bertrand et al. 2006b).

Female choice for more colourful males

Since carotenoid supplemented males had more saturated colouration, we expected that females would prefer them. As predicted, females spent significantly more time in association with β-supplemented-males than control males, indicating a preference for more colourful males. This is in accordance with a few previous studies performed in species with carotenoid-based plumage colouration (Hill 1990; Johnson et al. 1993; Hill 1994; Sundberg 1995; reviewed in Hill 2006; Leitão et al. 2014). Mate choice based on carotenoid ornaments could provide both direct and indirect benefits to females. It makes evolutionary sense if colouration signals good genes or healthy males. Good genes models propose that females gain indirect benefits by choosing males by an indicator of quality (Evans et al. 2004), improving their offspring fitness. Females can also have direct benefits, through parental care. Another benefit with both a direct and indirect component is that they could choose to mate with less parasitized males (Figuerola et al. 2003), or which are healthier. In our study, females would benefit by choosing more colourful males which had a higher immune condition.

In conclusion, our results support the hypotheses that carotenoid-based ornamentation is an honest sexual signal, encoding information about pigment access, nutritional condition and health. These results support an indirect signalling role for yellow carotenoid colouration, in accordance with previous suggestions (Hartley and Kennedy 2004). They also agree with the vitamin A redox hypothesis, through an indirect way (Hill and Johnson 2012). Further work should try to understand the mechanisms than maintain this association.

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ETHICAL STANDARDS

All experiments were performed in accordance to Portuguese legislation for research on animal behaviour, and were conducted under license permits: 258/2009/CAPT to PGM and 259/2009/CAPT to ST, by Instituto da Conservação da Natureza e da Biodiversidade (ICNB).

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CHAPTER 3

Age and parasites predict carotenoid-based plumage colour on male European serin

ABSTRACT

A fundamental assumption of theories on the evolution of sexual signals is that they should be costly to produce and honestly signal the quality of the sender. The expression of carotenoid-based plumage signals are thought to be condition-dependent traits, due to carotenoids function as pigments and health modulators.

We explored carotenoid-based plumage colouration in a free living population of male European serins, *Serinus serinus* during the breeding season. Male serins were trapped for morphometric and colorimetric measurements, during a four-year field study, in order to evaluate the signalling value of colouration in relation to body condition and level of parasites. We started by evaluating two different types of colorimetric measurements: the most commonly used tristimulus colour variables, based on human colour perception, and the physiological models of avian colour vision, and found that they were highly correlated. Secondly, we investigated which factors influenced the expression of plumage colour and we found that plumage colour expression was influenced by age and ectoparasite load. Our results indicated that the colour expression of the plumage of the serin is an age dependent trait and an honest signal of the ability to cope with parasitic infection.

Keywords: parasites; condition; carotenoid-based ornamentation; *Serinus serinus*.

INTRODUCTION

Over the past decades a central theme of research among behavioural ecologists has been the mechanism, function and evolution of sexually selected traits in animals (Andersson 1994). It is well known that many species choose mates with well-developed ornaments, and that from this choice they can obtain direct or indirect benefits. One of the most studied sexually selected traits in birds is the colour conspicuousness. Birds could show colouration in different body parts. Plumage colouration is particularly widespread and can result from structural colours, interference in reflection and the deposition of melanin, carotenoids or other pigments (Hill and McGraw 2006).

In animals, bright yellow to red carotenoid-based colouration is a common ornamentation and these colours are good candidates for condition-dependence ornamentation, since carotenoids are mostly acquired by ingestion (Goodwin 1984). Condition could be defined as the property that confer great fitness to individuals and it can have genetic and environmental components (Hill 2011; Iwasa and Pomiankowski 1999) and honest signalling may be maintained because only high-quality individuals can pay the costs of producing and maintaining the trait (Zahavi 1975). A positive link between the expression of the ornament and indices of condition is assumed, such as body condition or immunocompetence. Since carotenoids have antioxidant and immunoregulatory functions, more colourful individuals may signal their health through this signalling (McGraw 2006; Peters 2007). Choosing more ornamented mates could be advantageous due to some heritable component or viability, providing indirect benefits or good genes. Mates could also benefit from direct advantages, like access to resources or parental care. Bright colouration in birds could also reliably indicate genetic resistance to parasites (Hamilton and Zuk 1982). Parasites can increase the risk of infestation of the mate (Hillgarth 1996; Milinski and Bakker 1990) and might affect production of ornamental colouration. The Hamilton-Zuk hypothesis proposes that

coloured traits function as reliable indicators of resistance to parasites (Hamilton and Zuk 1982), as parasitized males will show decreased expression of the secondary sexual traits preferred by female. Previous work show some evidences for this hypothesis (Brawner et al. 2000; Hamilton and Zuk 1982; Harper 1999; McGraw and Hill 2000b; Møller, Christe and Lux 1999; Thompson et al. 1997).

We conducted a field study to investigate whether body condition, parasite load, and age affected carotenoid-based plumage colouration in the European serin, *Serinus serinus*. We assess the individual colour variation of serins in a natural population, during pair formation and breeding. The serin is a small sexually dichromatic cardueline finch, with males' displaying yellow carotenoid-based patches on crown and breast, which is sexually selected (Leitão et al. 2014). We predicted that carotenoid-based plumage is condition dependent, i.e., less parasitized males and in better body condition should be more ornamented. We also predicted that older males would have greater ornament expression than younger males.

An important factor in studies of sexually coloured traits is to accurately assess colour as birds perceive it. Avian vision is different from human vision in three main features: birds have a broader spectral range than humans (300-700nm for birds, 400-700nm for humans) (Cuthill et al. 2000; Hart 2001); avian colour cones contain coloured oil droplets, not found in humans; and avian eyes have four colour cones, whereas human eyes only have three colour cones (the presence of a fourth cone allows birds to perceive UV wavelengths) (Cuthill 2006). In the past, quantitative measurements of plumage colouration have been performed using a tristimulus score, based on human vision, described as hue, chroma or saturation and brightness (HSB). The use of tristimulus human vision based variables had raised some concerns about the signficicance of these measurements. One criticism is that frequently the UV-component of the bird visible spectrum is neglected, although UV plumage colours are taxonomically widespread in birds (Eaton and Lanyon 2003; Hausmann et al. 2003). Besides, as birds are tetrachromatic, trichromatic colour variables could not fully explain variation. Birds can see a greater diversity of colours than humans, because of

their higher capacity to discriminate colours (Vorobyev 2003). Therefore, analyses of avian colouration should consider the full extent of avian visual capabilities. We wanted to assess if these two different modes of quantification provided different classifications of the individual colouration. We used the tristimulus colour variables (Montgomerie 2006) and avian visual models derived from quantum cone catches (Stoddard and Prum 2008), taking into account the avian visual sensitivity.

METHODS

Morphometric measurements

We captured 100 male serins with mist-nets in agricultural fields nearby Coimbra, Portugal, from 2008 to 2012, between February and April. Birds were classified according to their age: 35 aged as 1st year, 62 aged as 2nd year or older and three with unknown age. Males were trapped with mist nets colour banded for individual identification, and measured. When a male was captured more than once in the same year, we averaged the recorded measurements. We measured body mass with a pesola balance (accuracy of 0.5 g) and tarsus length with a calliper (accuracy of 0.5 mm). Descriptive statistics for the morphometric measurements are given in Table S1 (Supplementary material).

As a measure of body size we used the score of a principal component (PC) that best represents body size, from a principal component analysis (PCA) of untransformed morphological measurements. The PCA for morphological measurements revealed one PC with eigenvalues larger than one, characterised for positive loadings of all variables and explaining 38% of total variation (trait loadings: wing length 0.751; tarsus length 0.292; mass 0.702).

Parasite load

Our model species, the serin, is infected by the feather mite *Proctophyllodes serini*. We inspected the primaries of both wings for feather mites by holding the extended wing up to the ambient light. The number of feather mites on the primary feathers was recorded based on a four-point scale developed by Behnke et al. (1999; 1995): 0 (no parasites), 1 (few parasites along rachis), 2 (few parasites around rachis and some on barbs) and 3 (parasites cover all rachis and some on barbs). Ectoparasite load was calculated as the average of the two wings, after the sum of the scores for each primary.

Coccidians of the genus Isospora are common endoparasites of wild birds. These protozoans are intestinal parasites transmitted by a faecal-oral route. Intestinal parasite load was estimated by counting in faecal samples collected from birds (Mougeot et al. 2004). Birds were kept individually in a small paper bag for up to 30 min to collect faecal samples and were then released. The droppings produced by each bird were collected and stored in Eppendorf tubes at -20ºC until analysis. Each Eppendorf tube was filled with distilled water, mixed, and the droppings dissolved. The mixture was then placed in a MacMaster slide and let sit for 5 minutes before scanning under a microscope for coccidian eggs count. The number of oocysts present on each coverslip was recorded on a scale from 0 to 5: 0 (no oocysts), 1 (1 to 10 oocysts), 2 (11 to 100 oocysts), 3 (101 to 1000 oocysts), 4 (1001 to 10000 oocysts), and 5 (more than 10000 oocysts) (Brawner et al. 2000). We calculated the prevalence (number of birds infected/number of birds examined) and severity (mean infection score for infected birds only) of ectoparasite load.

Colour measurements

European serins are sexually dichromatic cardueline finches with males being yellow-bright and females' drabber (Cramp and Perrins 1994). The yellow patches are

due to canary xanthophylls A and B, presumed to be derived from lutein through dehydrogenation (McGraw 2006; Stradi et al 1995). Birds undergo one annual moult between July and November when their entire plumage is replaced before the reproductive season which occurs between late February and June.

We measured carotenoid-based colouration in four body parts: forehead, throat, breast and belly. Colour measurements were made using an Ocean Optics USB4000 spectrophotometer with a deuterium-halogen light source (DT-Mini-2-GS, Ocean Optics) and a Y-shaped probe (Ocean Optics, Dunedin, FL, USA) mounted in a holder that kept it at 3 mm from the feathers (28 mm^2 measuring area), from 300 to 700 nm. Measurements were taken perpendicular to the feathers 'surface and were calibrated against a white standard (Ocean Optics, WS-1-SS White Standard) that was scanned before measuring each bird. We measured each body part three times to account for possible heterogeneity of the colouration. We analysed spectral data using two different methods: colorimetric variables and avian vision models.

Each bird's plumage reflectance data was summarized by calculating tristimulus scores — hue, saturation and brightness. Hue was calculated as the wavelength reflection halfway between the minimum and maximum reflection values, saturation as the total reflectance in a region divided by the total reflectance, and brightness was calculated as the mean reflectance of the entire spectra (Montgomerie 2006) (Table 1). The mean colour variables were then averaged through the four areas. We used R studio to calculate these variables (R Development Core Team 2013).

Table 1 Colour variables used in the analyses of birds' colours. $\boldsymbol{R_{\lambda i}}$ represents the percentage of reflectance at $\boldsymbol{\lambda_i}$ (wavelength), $\boldsymbol{n_w}$ the number of wavelengths intervals used.

Colour variable	Formula
Hue (H)	$H = \lambda_{Rmid} = (R_{max} + R_{min})/2$
Saturation (S)	$S = (R_{max} - R_{min})/B$
Brightness (B)	$B = \sum_{320}^{700} R_i / n_w$

With visual modelling, colouration scores incorporate the receiver's visual sensitivity. The spectral data was summarized into four quantum cone catches, corresponding to the four single cones found in the avian retina (Cuthill 2006): ultraviolet (UVS), short (SWS), medium (MWS), and long wavelength sensitive (LWS) and the double-cone (DC). Following Vorobyev et al. (1998), we calculated cone quantum catch for each of the four avian cones as the summed product of plumage reflectance, the ambient illuminant, and the absorbance spectrum of the cone across the wavelengths of the avian visual spectrum (300 to 700 nm; equation 1 in Vorobyev et al. 1998). The model included standard daylight (D65), ideal (white) background, and the visual bird model. Since visual model for our species is not available, we used the blue tit *Cyanistes caeruleus* system (Hart et al. 2000), which is commonly taken as a representative of birds UVS vision (Håstad et al. 2005). SWS ratio was calculated as the ratio between the SWS cone and the mean of the other cone catches. To achieve normality, we used the natural logarithm of SWS ratio. Plumage colouration was scored by two independent variables: SWS ratio and double cone, representing chromatic and achromatic indices of plumage reflectance (Evans et al. 2010; Osorio et al. 1999). We used the software package PAVO, running in R (R Development Core Team 2013), developed by Maia et al. (2013).

In addition, we measured the size of the yellow patch by overlaying a transparent square grid over the patch and counting squares (Hill 1992). The grid was vertically positioned over the area and grid squares (9 mm^2) with yellow coloration were counted. We further converted number of squares in cm^2, and present the results as cm^2. All the measurements were performed by the same researcher (ST).

Descriptive statistics for the colouration measurements are given in Table S2 (Supplementary Material). We used generalized linear models (GLM) to test which factors were predictors of plumage colouration, using plumage ornament variables: achromaticity (double cone), chromaticity (SWS ratio) and patch size as response variables, assuming normal distribution of error term. Predictors in the models were age, ectoparasite load and body size (PCA of body mass, wing and tarsus length). We report Wald $\chi 2$ with respective p-values for significance. We further repeated this analysis with intestinal parasite load in a separated GLM, due to smaller sample size (n = 20).

Statistical analyses were performed by using IBM SPSS v21.

RESULTS

We found that the two colour metrics were highly correlated. In male serins, plumage achromaticity is highly positively correlated with brightness (Table 2). Plumage chromaticity is highly positively correlated with saturation and hue and negatively with brightness (Table 2). Patch size is positively correlated with hue (Table 2). There is considerable agreement between the two types of measurements for this species coloration.

Table 2 Pearson correlations between tristimulus and avian visual model colour variables, whit the coefficient of correlation and significance. * P < 0.05; ** P < 0.001; *** P < 0.0001.

	SWS ratio	Saturation	Hue	Brightness	Patch size
Double-cone	-0.202*	-0.165	0.077	0.988***	-0.041
SWS ratio		0.868***	0.279*	-0.319**	0.078
Saturation			0.141	-0.284*	-0.007
Hue				0.083	0.215*
Brightness					-0.039

The prevalence of ectoparasites (82%) and intestinal parasites (100%) was widespread, although the severity of infection was low (ectoparasites =6.85; maximum possible = 54; intestinal parasites =2.05; maximum possible = 5).

We detected that ectoparasite load and age were the main predictors for colouration variables. Plumage achromaticity was predicted by ectoparasite load, plumage chromaticity and patch size were predicted by ectoparasite load and age (Fig. 1d and 1e) (Table 3). Plumage achromaticity decreased with ectoparasite load as well as patch size, but chromaticity varied positively with ectoparasite load (Fig. 1a to 1c).

Body size PC and intestinal parasite load were not significant predictors for any of the models. GLM's with hue, saturation and brightness yield similar results (Table 3 of Supplementary Material).

Table 3 Generalised linear models showing the predictors of colour variables of plumage colouration, presenting the Wald χ^2 result and respective p-values for significance. d. f. stands for degrees of freedom; β for estimate of the model.

	β	Wald χ^2	P
Plumage achromaticity			
Age	-0.001	0.01	0.92
Ectoparasite load	-0.003	23.88	< **0.001**
Body size PC	-0.002	0.22	0.64
Intestinal parasite load	-0.003	0.33	0.57
Plumage chromaticity			
Age	-0.032	4.95	**0.03**
Ectoparasite load	0.005	13.96	< **0.001**
Body size PC	-0.001	0.01	0.92
Intestinal parasite load	0.027	1.30	0.25
Patch size			
Age	-0.464	4.29	**0.04**
Ectoparasite load	-0.063	8.96	**0.003**
Body size PC	-0.024	0.05	0.83
Intestinal parasite load	-0.095	0.06	0.81

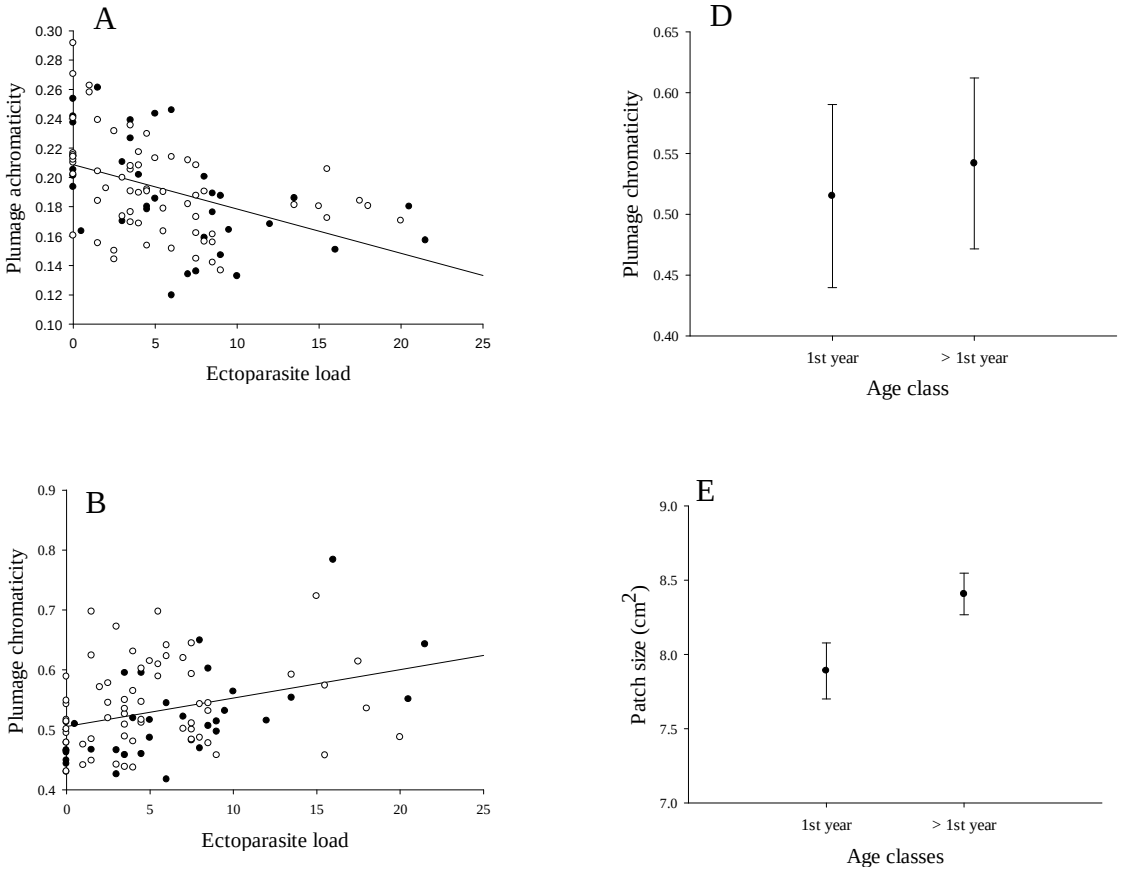

Figure 1 Predictors of the colouration variables. (A) plumage achromaticity; (B) plumage chromaticity and (C) patch size were predicted by ectoparasite load; close circles represent 1st year males and open circles represent > 1st year males. (D) plumage chromaticity and patch size (E) were predicted by age class.

DISCUSSION

Our results show that the tristimulus variables saturation and brightness were highly positively correlated with avian visual models variables (DC and SWS ratio). We found that plumage colouration could be predicted by ectoparasite load and age class, with less parasitized males presenting more achromatic and larger plumages patches. Older males had higher values of plumage chromaticity and had larger plumages patches than younger males. However, more parasitized males also had higher values of chromaticity.

Comparison between quantitative colorimetric models

Human and bird eyes have fundamental differences, as avian retinas have four cone types, which leads to the assumption that birds are tetrachromatic and a three dimension explanation of colour is not accurate (Cuthill 2006)., Avian visual models had been developed in recent years in order to account for the avian vision discrimination (Endler and Mielke 2005; Vorobyev et al. 1998). We found that these models were highly correlated with the human perceived tristimulus variables in the case of the yellow carotenoid coloration of male serins. In line with theoretical assumptions, achromatic variables are represented by brightness, which is the mean of the light reflected by a surface (Campenhausen and Kirschfeld 1998; Osorio et al. 1999), and chromatic variables are represented by chroma (or saturation) and hue (Evans et al. 2010). Tristimulus colour variables seem to capture the colour variation perceived by birds and it was demonstrated that the tristimulus colour variables are significantly correlated with carotenoid contents in mallard *Anas platyrhynchos* and house finch *Carpodacus mexicanus* feathers (Shawkey et al. 2006; Butler et al. 2011), so they seem to be a good human perceptive measurement.

We also found a positive correlation between patch size and hue, which points to a multiple component message. Patch width and colour were also positively correlated in

rock sparrow *Petronia petronia* (Griggio et al. 2007). Patch size is expressed by the number of follicle cells that are receptive to the uptake of carotenoid pigments during feather growth (Brush 1990) and can be affected by dietary carotenoid access (Hill 1992). Access to carotenoid pigments can influence the uptake of carotenoids into the follicle as well as the number of follicles that uptake the pigment, which result in a positive correlation between carotenoid expression and patch size (Badyaev et al. 2001; Hill 1992). This way, in a single trait, birds could be given information about two different mechanisms.

Trait value

Our results support the prediction that the abundance of parasites modifies the expression of sexual ornaments. Carotenoid-based plumage colouration should be particularly sensitive to parasite infections (Dawson and Bortolotti 2006; Hõrak et al. 2004; Lozano 1994; Mougeot et al. 2009) and birds with more mites tended to grow duller plumage (Harper 1999; Thompson et al. 1997) and have smaller patches (Vergara et al. 2012). Figuerola and colleagues (2003) had showed that in serin, ectoparasites load during moult affect negatively the plumage colouration after moult, specifically brightness and saturation. In our study, the measurements were taken in the beginning of reproductive season, several months before and after the next moult, and it was also found a negative relationship with chromaticity and achromaticity. Consequently, by choosing to mate with a highly ornamented male, females may get indirect benefits, in the form of parasite-resistance genes for her offspring and direct benefits, in the form of reduced risk of infestation.

Intestinal parasites did not predict any of the colouration variables, contrary to what we expected, since they could directly inhibit the uptake of carotenoids and other essential dietary components by damaging the epithelial cells of the host's intestine (Allen 1987; Hõrak et al. 2004). In other species, a negative effect of intestinal parasites

was observed on plumage colouration of house finches (Brawner et al. 2000), American goldfinches *Carduelis tristis* (McGraw and Hill 2000b) and greenfinches *Carduelis chloris* (Hõrak et al. 2004). The lack of relationship in the present study could be a result of a bias in our sampling protocol, since we collected faeces samples in the morning, which give estimates of both coccidian prevalence and load significantly different from afternoon in serin (López et al. 2007). Counting oocysts in host faeces is the only non-invasive method of determination of intestinal parasite load (Watve and Sukumar 1995). However, this method could be inaccurate for field studies due to the limitation of one sample per individual and circadian variation in oocyst shedding.

If the carotenoid-based plumage is an honest signal of quality, one may expect age and condition to predict saturation (Andersson 1994; Inouye et al. 2001). This is indeed the case for age, with older males having higher values of plumage chromaticity, which is highly correlated with saturation. In other species ornamental colouration increase with age (Siefferman et al. 2005) and in a meta-analysis, Evans et al. (2010) found that age is a highly significant predictor of all three tristimulus colour variables. Female preference for older males is a common occurrence (Richardson and Burke 1999; Sundberg and Dixon 1996) and mating success is correlated to age (Johnstone 1995). By choosing a more colourful male, females may be selecting older and experience males (Budden and Dickinson 2009). Females may gain good genes from older males (Manning 1985) or better resources (Marchetti and Price 1989). Nevertheless, older males being more colourful could result from a different process, as a higher mortality of less colourful animals (Hõrak et al. 2001), although Figuerola et al. (2007) have found a higher survival of intermediate coloured serins.

Additionally, we found that individuals with higher values of plumage chromaticity had more ectoparasites. This result is surprising, since we expected that plumage chromaticity revealed the ability to cope with parasitic infection. We know that females choose males which have more saturated plumage (Leitão et al 2014) and have results from a lab experiment (Chapter 2 on this thesis) which indicate that males that developed more saturated plumage had a higher capacity for immune response. Thus, if

more colourful males are healthier, why do they present more ectoparasites in the wild? There are some possible explanations for this. In the first place, our result indicates that ectoparasite load does not affect colour intensity, since the correlation was not positive. Secondly, we can assume that ectoparasite load is not particularly damaging to the health and physiological condition of individuals, although it affects the condition of the feathers, which is well expressed in the effect over the achromatic component of colouration. If so, males would not suffer much selection on avoiding ectoparasites. We know that females choose males based on colour saturation, but we do not know whether the achromatic condition of feathers is selected. Thirdly, because more colourful males are healthier they may be capable of supporting higher levels of ectoparasitism without being affected as much as the less colourful males. And fourthly, it is even possible that some of the lifestyles of more and less colourful males differ in aspects that make the more colourful males more susceptible to acquire ectoparasites. Variation in carotenoid colouration is mostly due to non-genetic factors (eg. Evans and Sheldon 2012), with condition and experience and access to food sources playing the major roles. So, individual differences in colour expression also reflect different abilities of individuals to find and process food.

We found that older and less parasitized males had a larger plumage patch size, which can indicate that patch size could also be a condition-dependent trait in this species. In birds, the colour of the plumage patches is usually sexually selected (Hill 2002) however the size of the patches may also be important (Badyaev et al. 2001). There are some species where the size of the patch is the main selected trait, as in males' rock sparrow *Petronia petronia* (Griggio et al. 2007) and collared flycatchers *Ficedula albicollis* (Sheldon et al. 1997). Nevertheless, patch size is usually associated with signalling status. For example, in red-collared widowbird *Euplectes ardens*, redness and size of carotenoid ornament indicate male dominance status (Pryke et al. 2001; 2002).

Carotenoid-based plumage colouration signals are generally considered as being condition-dependent in many species. However, feather signals are formed several

months prior to mate choice, which leads to the question of maintenance of the honesty of this trait. The honesty of the signal could also be maintained by another mechanism demanding condition-dependency, male-male competition. Carotenoid-based plumage colouration is thought to be sexually selected, but the role in male-male competition is not so clear, with contradictory results (McGraw and Hill 2000a; Pryke et al. 2001). Our results support the hypothesis that carotenoid-based plumage colouration is condition-dependent, with high-quality individuals being more conspicuous. Besides, carotenoid ornamentation reflects capacity to cope with parasitic infection.

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SUPPLEMENTARY MATERIAL

Table S1 Descriptive statistics of morphological measurements and parasite load, for males' serins. SE sands for standard error, CV stands for coefficient of variation, N = 100.

	Mean ± SE	CV (%)
Wing length (mm)	70.55 ± 0.168	2.4
Tarsus length (mm)	15.18 ± 0.143	9.4
Mass (g)	11.53 ± 0.092	7.9
Ectoparasite load	5.68 ± 0.513	89.8
Intestinal parasite load	2.05 ± 0.129	28.8

Table S2 Descriptive statistics of colouration measurements for males' serins. SE sands for standard error, CV for coefficient of variation. N = 100.

	Mean ± SE	CV (%)
Plumage achromaticity	0.191 ± 0.003	18.0
Plumage chromaticity	0.53 ± 0.007	13.7
Brightness	15.67 ± 0.300	19.1
Hue	495.65 ± 2.758	5.6
Saturation	1.49 ± 0.013	9.0
Patch size (cm^2)	8.22 ± 0.131	13.8

Table S3 Generalised linear models showing the predictors of colour variables of plumage colouration, presenting the Wald χ^2 result and respective p-values for significance. d. f. stands for degrees of freedom; β for estimate of the model.

	d. f.	β	Wald χ^2	P
Hue				
Age	93	-4.328	0.565	0.452
Ectoparasite load	93	-0.870	2.590	0.108
Body size PC	93	-4.192	2.222	0.136
Intestinal parasite load	18	0.952	0.009	0.925
Saturation				
Age	93	-0.064	6.097	**0.014**
Ectoparasite load	93	0.010	15.854	< **0.0001**
Body size PC	93	-0.009	0.519	0.471
Intestinal parasite load	18	0.060	1.686	0.194
Brightness				
Age	93	0.186	0.111	0.739
Ectoparasite load	93	-0.289	30.439	< **0.0001**
Body size PC	93	-0.132	0.234	0.629
Intestinal parasite load	18	-0.397	0.629	0.428

CHAPTER 4

A test of the effect of testosterone on a sexually selected carotenoid trait in a cardueline finch

ABSTRACT

A great number of secondary sexual traits are assumed to have evolved as honest signals of individual quality. It is known that androgens regulate many male secondary traits as well as reproductive behaviour. The expression of melanin-based colouration is modulated by androgens, particularly testosterone, and there is some evidence that carotenoid-based colouration may also be under androgen control. In the European serin, *Serinus serinus*, male carotenoid-based plumage colouration is a sexually selected trait, subjected to female choice. In this experiment, we investigated if testosterone influences the expression of this trait by manipulating testosterone levels during moult and assessing how it affected plumage colour expression after moult. We found that testosterone had only a negative effect on the size of the yellow ornament. Our experiment shows that testosterone had a limited effect on carotenoid-based colouration of a cardueline finch.

Keywords: testosterone; carotenoid; ornamentation; European serin; carotenoid-based colouration.

INTRODUCTION

Males frequently have elaborated secondary sexual traits, which are used both in mate attraction or male-male competition, and females often select males using these exaggerated traits (Andersson 1994). Conspicuous ornaments can signal individual quality (Hamilton and Zuk 1982; Zahavi 1975) or condition and health (Barron et al. 2013; Griggio et al. 2010; Svobodová et al. 2013), and they are assumed to have evolved due to direct or indirect benefits for females by mating with better quality or fitter males. In order to be maintained as signals of quality, these traits have to be evolutionarily honest, that is, they should be costly to produce (Zahavi 1975) and these costs may result from mechanisms that mediate allocation strategies, or are a consequence of trade-offs between different functions (Muehlenbein and Bribiescas 2005). Hormones, such as testosterone (T), have been considered to be mediators of those trade-offs in male vertebrates (Hau 2007; Ketterson and Nolan 1992), since the production of some secondary sexual traits could involve the costs associated with higher T levels. These costs can be the increase of basic metabolic rate (Buchanan et al. 2001), level of stress hormones (Ketterson and Nolan 1992) or the decrease of immunity (Casto et al. 2001; Mougeot et al. 2004; Verhulst et al. 1999). Thus, T has been considered a key factor in the concept of honesty of sexually selected signals.

The immunocompetence handicap hypothesis (ICHH) suggests that sexually selected ornaments and immune system compete for resources, meaning that while T controls for the expression of an ornament it has a negative effect on the immune system (Folstad and Karter 1992). There is some evidence supporting this assumption (Muehlenbein and Bribiescas 2005; Owen-Ashley et al. 2004; Roberts et al. 2004). An alternative hypothesis, the oxidation handicap hypothesis (OHH), proposes that T mediates the trade-off between the ornament expression and the resistance to oxidative stress (Alonso-Alvarez et al. 2007; Alonso-Alvarez et al. 2008). Experimentally elevated T levels affects red blood cell resistance to free radicals, indicating that high T

levels required for the expression of secondary sexual traits can cause increased oxidative stress (Alonso-Alvarez et al. 2007).

In birds, androgens, such as testosterone, control song, sexual ornaments and sexual and social behaviour (Mougeot et al. 2003; Zuk et al. 1995). In particular, it is known that T affects several aspects of the behaviour of birds, such as parental care, singing behaviour and aggressiveness, as well as physiological aspects such as metabolic rate, lipid storage and moult (reviewed in Ketterson and Nolan 1992). While sexual dimorphism in plumage is generally not modulated by T (Kimball 2006; Owens and Short 1995), the strength of the signal can be (Roberts et al. 2004), existing experimental evidence that T is responsible for regulating individual differences in the expression of secondary sexual traits in birds. The expression of melanin-based colouration is mediated by androgens (Bókony et al. 2008), for example, T is responsible for the bib size of male house sparrow *Passer domesticus* (Buchanan et al. 2001; Evans et al. 2000; Gonzalez et al. 2001). And, T can also play a role in carotenoid-based colouration. Male birds treated with T had increased circulating lipoproteins, which are plasma carriers for carotenoids, and increased bio-availability of carotenoids (Blas et al. 2006; McGraw et al. 2006; McGraw and Parker 2006). This result points to an integrative mechanism for controlling sexual traits. Carotenoids are responsible for the bright red, yellow and orange colouration of integuments in birds (Hill 1991; McGraw and Ardia 2003; Olson and Owens 1998), and they also enhance the immune response and have antioxidant functions (Blount et al. 2003; Faivre et al. 2003; Olson and Owens 1998). It was thus hypothesized that both androgens and carotenoids have a major function in the expression of honest signals of individual quality (Blas et al. 2006; Peters 2007), relevant both in sexual and social contexts (Blount et al. 2003; Faivre et al. 2003; Hill 1990, 1991; Lyon and Montgomerie 2012; McGraw and Ardia 2003; Zuk et al., 1995). T affects the comb size of red grouse *Lagopus lagopus scoticus* (Mougeot et al. 2004), the development of nuptial plumage in superb fairy-wrens *Malurus cyaneus* (Peters et al. 2000), as well as of the red-backed fairy-wren *Malurus melanocephalus* (Lindsay et al. 2011). However, no effect of T was

found on carotenoid-based plumage colouration of blue tits *Cyanistes caeruleus* (Peters et al. 2012) and old males of red-legged partridges *Alectoris rufa* (Alonso-Alvarez et al. 2009).

European serins *Serinus serinus* are dichromatic monogamous cardueline finches, with carotenoid-based ornamentation. Male serins have a distinct yellow patch which is sexually selected (Leitão et al. 2014). It is not known whether this trait which is produced during the single annual post-breeding moult is modulated by T. Moult is an challenging period for a bird, with high physiological costs: the daily energy expenditure at the peak of moult could be two to three times the basal metabolic rate (Lindström et al. 1993); thermoregulatory ability decreases due to impaired feather insulation (Klaassen 1995); and predation risk increases due to impaired flight abilities (Swaddle and Witter 1997). In this demanding period, even small differences in T levels could affect signal expression on plumage. From the few studies done so far on the relationship between T and carotenoid-based colouration, it is unclear whether an effect of T does exist on this type of colouration. We wanted to determine if there was one in relation to the carotenoid-based colouration of male serins. Therefore, we experimentally tested if T-levels affect the expression of carotenoid-based plumage colouration by manipulating T in males undergoing annual moult. T should enhance sexual ornamentation with a concomitant decrease in body condition. If that is the case in the serin, we expect that higher plasma T levels will contribute to an increase in carotenoid-based yellow patch and to a decrease in physical condition.

METHODS

Experimental design

We captured 32 male serins during January 2012 in agricultural fields nearby Coimbra, Portugal (40° 19'; -8° 58'). Birds were kept in groups of 4, in 8 cages (90 x 50 x 40 cm), in an indoor aviary at the Department of Life Sciences of University of Coimbra and released at the end of the experiment (November 2012). They had ad libitum access to a commercial seed mixture, water and grit. The aviary had natural light and ventilation, allowing the birds to be exposed to the natural photoperiod and moult in natural conditions. We banded birds with a numbered black ring and measured their morphometry and plumage colouration both after capture and after moult. We also took blood samples to assess plasma T levels at three different times: before, during and after moult (February, August and October 2012). Body mass was measured with a pesola balance (accuracy of 0.5 g) and tarsus length with a calliper (accuracy of 0.5 mm).

We collected blood samples from brachial vein; the plasma was centrifuged, extracted, and kept at -20ºC until used for hormone analyses by radioimmunoassay (RIA). All samples were analysed in duplicate. Intra-assay variation was 2.4% and 4.5%. The inter-assay variation was of 11.5%. T was undetectable in 11% of 90 samples. Blood plasma steroid extraction was done using a previously described method (Canario and Scott 1989). Steroid residues were re-dissolved in phosphate buffer 0.1 M, pH 7.6, containing gelatine (1 g/L), and stored again at -20°C until assayed for T. Radioimmunoassay were conducted using a T reactive marker from Amersham Biosciences (specific label ([1,2,6,7-3H] Testosterone, ref. TRK402-250mCi) and T antibody from Research Diagnostics Inc. (ref: WLI-T3003). Total androgen concentrations are reported as ng/ml.

Colouration measurements

We took measures of the plumage colouration of male serins in four different areas: forehead, throat, breast and belly, before and after moult. Reflectance (R) of each area was measured by sampling three times in each region and averaging them, with an Ocean Optics USB4000 spectrophotometer attached to a deuterium and halogen light source (Mikropack Mini-DT-2-GS, UV–VIS–NIR), emitting between 300 and 700 nm (Figure 1). All measurements were taken relative to a WS-1-SS white standard (Ocean Optics). The probe (Ocean Optics R400-7 UV–VIS) was held vertically at a standardized distance, using a holder that provides a sampling area of 28 mm^2. We summarized plumage colouration in four colour metrics in visible (420-700 nm) and UV wavelengths (320-420 nm): 1) Brightness ($B = \sum_{320nm}^{700nm} R/n$); 2) Hue ($H = \lambda_{Rmax}$), 3) Saturation ($S = R_{320-700nm}/B$) and 4) UV-Chroma ($UVC = R_{320-400nm}/B$) (Montgomerie 2006). The mean colour variables were averaged through the four areas.

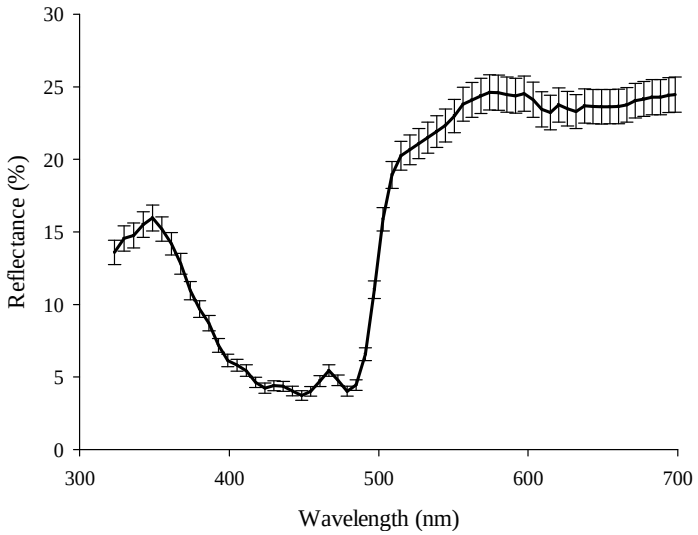

Figure 1: Reflectance spectrum from the yellow carotenoid-based plumage of European serins *Serinus serinus*. Mean ± standard error of raw data from the breast of males (N = 27).

Colour patch size was measured by overlaying a transparent grid over the patch and counting the number of squares (9 mm^2 each) that overlaid the yellow colouration. We then converted the number of squares into an area, and present the results in cm^2. For the breast, the transparency was vertically positioned in a way that the central line was centred with the bird and the top was positioned at the base of the lower mandible of the bird (Hill 1992). For crown, the transparency was positioned over the head centred with beak. All the measurements were performed by the same researcher (ST).

Hormone assays

For this experiment we choose to perform hormone implantation, without gonadal removal. We estimated implant sizes (5 mm Silastic Tubes, i.d.= 0.76 mm, o.d.= 1.65 mm Dow Corning) based on studies in other passerine species (Fusani 2008; Roberts and Peters 2009; Soma et al. 2000; Tramontin et al. 2003; Van Hout et al. 2011).

On 1st August 2012, 32 males were randomly assigned to a control group (control males), and were implanted with an empty tube or to a T implanted group (T-treated males), and were implanted with a T-filled tube. On the day before, we washed the silastic tubes with ethanol, filled half of them with T (T, product number 86500, Sigma-Aldrich) and closed both ends of the tubes with sterile silicone. The tubes stayed overnight embedded in PBS. For the implantation, one person held the bird while another person made a small incision in the upper layer of the skin on the back, on the neck, inserted the tube and closed the incision with veterinary glue (Vetbond, 3M, USA). Birds received local anaesthesia (Nexcare, Coldhot Cold Spray, 3M) before the surgery. Following implantation, birds were immediately released into their housing aviary. We observed birds in the next few hours and through the following day, revealing normal activity and behaviour. Twenty days later we collected blood samples to measure plasma T levels. The 32 males were randomly housed in 8 cages**.**

Statistical analysis

Physical condition was estimated as the unstandardized residuals of a linear regression of weight on tarsus. The relationship between the two variables was linear, with residuals over tarsus having an even distribution (Schulte-Hostedde et al. 2005). Before treatment there was no difference between the two groups in plasma T levels, physical condition, plumage colouration, and patch size (Table 1).

Table 1: Descriptive values (mean ± standard error) of plasma T levels (ng/ml), physical condition and colouration variables and F values for the ANOVA for differences between the two groups (T-treated males and Control males) before treatment (N = 27) and respective p-values for significance.

	Control males	T-treated males	F	P
Plasma T level	0.32 ± 0.043	0.40 ± 0.047	1.20	0.29
Brightness	16.18 ± 0.524	16.06 ± 0.602	0.02	0.88
Hue	531.88 ± 3.657	538.09 ± 2.507	1.90	0.18
Saturation	1.33 ± 0.015	1.34 ± 0.022	0.44	0.51
UV-Chroma	0.62 ± 0.015	0.66 ± 0.0144	3.16	0.09
Patch size	7.48 ± 0.225	7.63 ± 0.361	0.13	0.72
Physical cond.	0.008 ± 0.223	-0.009 ± 0.259	0.003	0.96

For the analysis of the effect of T manipulation on plasma T levels, plumage colouration and physical condition we performed a repeated measures ANOVA. The data was normally distributed (Kolmogorov-Smirnov test $p > 0.05$) for the three moments. There was an effect of time on plasma T levels ($F_{(2, 26)} = 11.440$, $p < 0.001$, Observed power= 0.986). However, neither the effect of treatment nor the interaction between treatment and time was significant (Treatment: $F_{(1, 13)} = 0.170$, $p = 0.687$,

Observed power= 0.067; time x treatment: $F_{(2, 26)}$= 0.228, p = 0.798, Observed power= 0.082). This was due to the fact that T levels also increased in the control group during moult (Fig. 2).

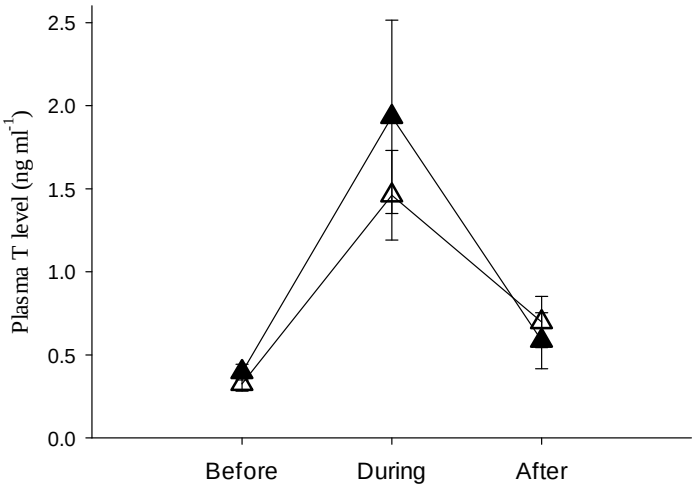

Figure 2: Comparison of plasma T levels between control (open circles) and T-treated males (closed circles) before, during and after moult (values are mean ± standard error).

Therefore, since the treatment and control groups did not differ significantly in T we performed generalized linear mixed models, with individual T-levels as independent predictors, while also including treatment (T implanted and control) and cage as factor. We used general linear mixed models using maximum likelihood (ML) to conduct F tests for the effects of plasma T levels on physical condition and plumage expression. Cage number where the birds were kept was included as a random factor. The variables were normally distributed and homogenous and the errors of regressions were independent. All analyses were performed using IBM SPSS Statistics® 21.0 for Windows.

RESULTS

Individuals with higher plasma T levels moulted into plumages with smaller yellow patches (p= 0.033) (Table 2 and Fig. 3).

Table 2: Results of F tests of general linear mixed models testing for an effect of plasma T levels on plumage colouration variables and physical condition, after moult. Models included cage number as a random effect. T treatment (T implanted and control) was included as a factor. Values are F values (F) and probabilities (P).

	Intercept		Plasma T level		Treatment	
	F	P	F	P	F	P
Brightness	1148.6	<0.001	0.18	0.68	1.71	0.21
Hue	11140.4	<0.001	0.70	0.41	0.70	0.41
Saturation	3040.1	<0.001	0.54	0.47	3.57	**0.07**
UV-Chroma	1512.3	<0.001	2.86	0.10	15.07	**0.001**
Patch size	983.8	<0.001	5.15	**0.033**	0.15	0.70
Physical cond.	1.292	0.267	1.42	0.24	0.10	0.76

Although there were no differences in plasma T levels between the two treatments, treatment had a negative effect on UV-Chroma (Control males= 0.67 ± 0.015; T-treated males= 0.61 ± 0.018; p= 0.001) and revealed a no significant tendency for lower saturation in T-treated males (Control males= 1.22 ± 0.016; T-treated males= 1.16 ± 0.026; p= 0.071). The other colouration parameters, brightness and hue, as well as physical condition were not dependent on the plasma T levels or T treatment (Table 2).

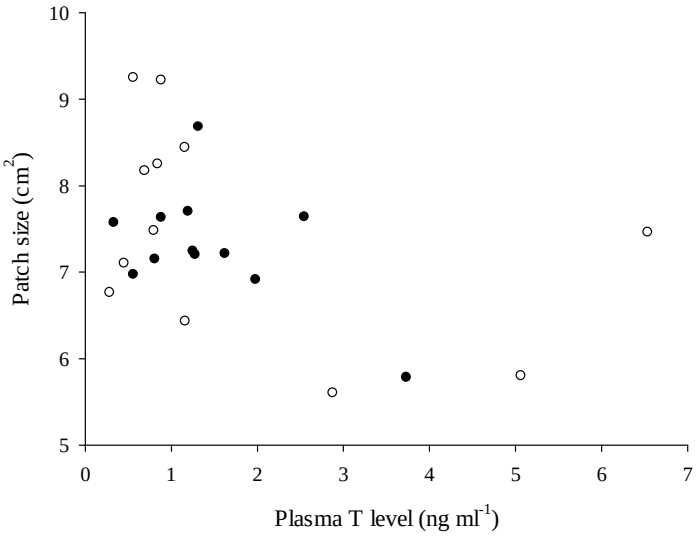

Figure 3: Negative relation between post-moult patch size (cm^2) and plasma T level during moult for control (close circles) and T-treated males (open circles).

DISCUSSION

We found that when comparing the T levels of all individuals, males with higher T values moulted into plumages with smaller yellow patches and T-treated males moulted into plumage with lower values of UV-Chroma. As expected there was a plasma T peak during moult in the T-treated males. However, our control males also experienced an increase in T levels which was unexpected. Male birds normally present relatively low levels of circulating T outside the breeding season (Dawson 1983; Hirschenhauser et al. 1999; Potter and Cockrem 1992; Wingfield 1984). The increase in T in non-treated males may be a consequence of the conditions of captivity. Roberts and colleagues (2009) also observed a similar peak in moulting birds in aviary, and they speculate that it was an artefact of captivity. However, the increase was so significant in our untreated

males that we favour a different explanation. It is quite likely that T-treated males became more aggressive due to the increase of T levels, and since non-treated males were housed in groups with T-treated males, this may have caused an elevation of T levels in the control males as a response to the increase in aggressiveness in their group. There are several sources of variability of T levels among male birds: individual mating opportunities, social status, aggressive encounters and other social interactions (including male–female interactions) (Kempenaers et al. 2008). The challenge hypothesis predicts that T levels will increase as a consequence of male-male competition (Wingfield et al. 1990). In laboratory, T levels of males increased when challenged by intruders (Wingfield and Wada 1989) and it could be influenced by social environment (Lacava et al. 2011).

The finding that T-treated males moulted into plumages with lower values of UV-chroma and that males with higher T values moulted into plumages with smaller yellow patches indicates that probably there is a cost for maintaining higher levels of T during moult. This is also supported by the non-significant tendency for reduced saturation in the T treated males. Our results suggest that males, in order to compensate for the negative effect of higher T concentration were forced to reduce the extension of their plumage signal. We know that in this species male yellow plumage saturation, which is an indicator of carotenoid concentration, is sexually selected (Leitão et al. 2014). Also, saturation is not naturally correlated with the size of yellow patch (Table S1 Supplementary Material). If the size of the patch is not sexually selected or is in a much lesser degree, males could strategically reduce the size of the signal while maintaining its intensity (saturation), which would counterbalance the costs of higher T-levels by affecting the least the expression of the signal. On other species no effects were found of T concentration on the expression of colouration on sexually selected plumage traits or bare parts. In zebra finches, no effect of T was detected on the expression of beak colour (Alonso-Alvarez et al. 2007); in male golden-collared manakins *Manacus vitellinus* T treatment increased display behaviours but did not affect feathers colouration (Day et al. 2006). In other cases the effect was only manifested in the long

term: in blue tits a positive relation was observed between crown UV reflectance and T, but only in the following spring, while no effect of T implantation was observed immediately after moult (Roberts et al. 2009).

The reduction of patch size in male serins with higher T values could also result from increased social competition, due to the high levels of testosterone during moult (Fig. 1) which would impose costs in the development of the signal. It is known that higher levels of intra-sexual competition can contribute to reduce the expression of secondary sexual traits (Martínez-Padilla et al. 2014), probably due to individuals adjusting the signal to their competitive ability. In red grouse, *Lagopus lagopus scoticus*, Vergara et al. (2012) found that comb size negatively correlated with population density, parasite infection levels, and climate conditions.

Although there were no differences in plasma T levels between the two treatments groups, when we included treatment in the analysis we found that T-treated males had lower values of UV-chroma and a tendency form lower saturation. The reduction in UV-chroma could be explained by a decrease of the quality of the feathers. UV reflectance depends mostly of the structural properties of the feathers (Shawkey and Hill 2005). An additional effect of T on the deposition of carotenoids in feathers may exist, but as the effect was not significant only further analysis would allow determining whether that is the case or not.

We did not detect an effect of T on males' physical condition. A decrease in physical condition was expected since it is generally considered that T acts as a mediator for trade-offs between physiological functions and signal production (Hau 2007; Ketterson and Nolan 1992). It is possible that due to aviary maintenance, all birds were in relatively good conditions, so that no effect of T was visible. We used a simple measure of condition which does not cover the many aspects in which condition can be impaired or decreased, although it was used with good results in other studies (Mougeot et al. 2004). The lack of visible effect of T on condition was reported in other studies: in red-winged blackbirds (Weatherhead et al. 1993), dark-eyed juncos *Junco hyemalis*

(Casagrande et al. 2011), blue tits *Parus caeruleus* (Foerster et al. 2002) and yellow-legged gulls *Larus cachinnans* (Alonso-Alvarez et al. 2002).

In conclusion, we show that T decreases the area of yellow carotenoid patch in male serins, and the T treatment decreases the structural reflective properties of feathers. However, T levels do not appear to affect male physical condition. Our results do not support any of the predictions derived from both the ICHH and OHH hypotheses, since higher T levels had no effect on physical condition and had a negative effect on the size of the colouration signal and the quality of the feathers. We additionally raise the possibility that males reduce their investment in less costly components of the signal, such as it's' extension in order to compensate for the costs of increased T levels.

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SUPPLEMENTARY MATERIAL

Table S1 Pearson correlations and significance values of saturation with UV-Chroma and patch size assessed in a sample of male serins, caught between 2007 and 2011, at agricultural fields nearby Coimbra, Portugal (n = 113).

	UV-Chroma	Patch size
Saturation	0.893 (< 0.0001)	-0.106 (0.263)
UV-Chroma		-0.062 (0.516)

CHAPTER 5

What does female carotenoid-based plumage colouration signal?

ABSTRACT

In species with male sexually selected colouration it is not immediately obvious why female ornamentation is also present. It could be the result of sexual selection action on females, or due to social selection on those signals, or resulting from natural selection, or be a non-functional by-product of selection on male ornaments. Examples for some of these possibilities were found in birds. In any case, the function of this kind of female ornaments can only be determined through investigation. European serins have dichromatic carotenoid-based plumages, with males exhibiting a large yellow patch in throat, breast and belly. Females present a much drabber colouration than males, but still exhibit some in yellow colouration, which can vary considerably. We tested for a possible function of female plumage colouration by performing a male mate choice experiment and a social competition experiment for access to limited food. None of the predictors for plumage colouration that we considered revealed any effect. We found no evidence for sexual discrimination by males based on female colouration. Furthermore, although female groups established steep hierarchies, dominance over food was not related to colouration. These results suggest that in this species, female plumage colouration is probably the result of genetic correlation with the males' selected trait.

Keywords: carotenoid-based colouration; female ornamentation; mate choice; social dominance; sexual selection; social selection

INTRODUCTION

The extraordinary traits that males of many species exhibit have been extensively studied (Andersson 1994; Darwin 1871), and a sexual or social signalling function had been attributed to them (Kraaijeveld et al. 2004). This has been particularly the case of colouration and song in male bird species. By contrast, female elaborate ornamentation has received much less attention although it is common its occurrence in females (Amundsen 2000; Cain and Ketterson 2012; Jones and Hunter 1993).

The evolution of female ornaments was initially explained by the Genetic correlation hypothesis, which states that female ornamentations are non-functional by-products of selection over male traits and kept in females by genetic correlation (Lande 1980). Other hypotheses suggest that female ornamentation could evolve as a result of sexual selection (Amundsen 2000) or broadly due to social selection (Tobias et al. 2012; West-Eberhard 1979; West-Eberhard 1983). The Mutual selection hypothesis states that female traits could result from similar processes to those working on males (Amundsen 2000; Johnstone et al. 1996; Kraaijeveld et al. 2007), i.e. males may also select for partners, and thus females compete for the attention of males (Amundsen 2000). Kraaijeveld and colleagues (2007), restated the idea advanced by Trivers (1972), that mutual mate choice may be common, particularly if both sexes provide parental care. The sex with more parental investment should be more selective than the less investing sex (Burley 1977). But when both sexes perform parental care, even the less investor sex is expected to exert some degree of mate choice (Bateson 1983). In birds, males are commonly the most ornamented sex (Andersson 1982), exhibiting exaggerated traits while females are often much less conspicuous, due to the fact that females are the choosing sex. But, males could also choose females by their ornamental colour traits. Males of several species have been shown to prefer more ornamented females, e.g., rock sparrow *Petronia petronia* (Griggio et al. 2005), burrowing parrots *Cyanoliseus patagonus* (Masello and Quillfeldt 2003), blue tits *Parus caeruleus* (Andersson et al.1998; Hunt et al. 1999), starlings *Sturnus vulgaris* (Komdeur et al. 2005), blue-footed

booby *Sula nebouxii* (Torres and Velando 2005), northern cardinals *Cardinalis cardinalis* (Jawor et al.2003), bluethroat males *Luscinia s. svecica* (Amundsen et al. 1997) and house finches *Carpodacus mexicanus* (Hill 1993). As it happens with females, males could choose females by quality, related physical attributes or by the outcome of female-female competition (Jones et al. 2001). However, in other species there was no signal that mutual mate choice was present for the ornaments tested (Murphy 2008; Tella et al. 1997; Van Rooij and Griffith 2012).

A third hypothesis to explain the evolution of female ornamentation is the Social selection hypothesis, that states that female ornamentation main function is as aggressive signal in social competition or for ecological resources rather than for mating (Tobias et al 2012; West-Eberhard 1979, 1983). Those ecological resources could be food, territories, nesting material, parental care or a place to sleep. In this sense, social selection includes any social interaction whether it is sexual or non-sexual. In group-living species, where individuals regularly interact, traits like colouration could serve as status signals, promoting social stability (Murphy et al. 2009a; West-Eberhard 1983). Status signals are expected to evolve in both males and females, when ornament evolution is due to social selection (West-Eberhard 1983), and both sexes compete for mating and non-mating resources. Status signalling is widely recognised to have evolved in males of many avian species (reviewed in Senar 2006). However, only a few studies have focused on the possible function of female ornaments such as in aggressive behaviour by signalling fighting ability (Griggio et al. 2010; Midamegbe et al. 2011; Murphy et al. 2009a; Murphy et al. 2009b; Swaddle and Witter 1995). In addition, a few studies have shown the existence of a possible dual function of female ornamentation (Griggio et al. 2010; Kraaijeveld et al. 2004).

Carotenoid-based plumage colouration is a sexual selected trait in many male bird species and it is thought to be an honest condition dependent signal (review in Olson and Owens 1998). It has also the potential to signal dominance status or fighting ability, with empirical works describing it but mainly in males (Griggio et al. 2007;

Murphy et al. 2009a; Murphy et al. 2009b; Pryke and Andersson 2003; Pryke et al. 2001). Female carotenoid ornamentation has also the potential to signal in mate choice choice context as well as competition for mates, territories, and other limiting resources (reviewed in Amundsen 2000; Kraaijeveld et al., 2007; Tobias et al., 2012).

The European serins (*Serinus serinus*) is a dichromatic species where individuals have a carotenoid-based plumage colouration, with males exhibiting a large yellow breast patch which is a sexually selected ornament (Leitão et al. 2014) and is also a status signal among males, along with plasma testosterone levels (Leitão, unpublished data). Females also present a yellow patch which is less defined and visible than in males, and which can be quite variable among females. This patch is absent in juveniles (Cramp and Perrins 1994).

In this study we wanted to determine the possible function of plumage colouration in female serins, both as a sexual or as a social signal in the context of mate choice and in a social contest over food. We performed a male mate choice experiment in a laboratory setup where males were able to choose between a more colourful or a drabber female. We also tested for intrasexual competition function, by creating a context of competition for access to limited food in newly formed groups of females. We attempted to test four hypotheses: 1) if female colouration is a sexual and a social signal, more colourful females will be preferred by males and colourful females will be more dominant; 2) if female colouration is a sexual signal, more colourful females will be preferred by males, but will not be discriminated in intrasexual context; 3) if female colouration is a social signal, more colourful females will not be discriminated by males but the signal will determine the outcome in competitive situations; 4) if female colouration is derived by genetic correlation of the males' trait, more colourful females will not be discriminated by males and will not be related with the outcomes of competitive situations.

METHODS

We captured 40 individuals (20 females and 20 males) with mistnets in agricultural fields nearby Coimbra, Portugal, between January and March of 2012, and transported immediately to an indoor aviary in the Department of Life Sciences of the University Coimbra where they were kept until the end of the experiments. Birds were aged (first year or older) and sexed as described by Svensson (1992), and received a numbered black plastic ring. Individuals were kept in same-sex groups of 4 per cage (118 x 50 x 50 cm), under natural light and ventilation, with several feeders and ad libitum access to a commercial food mixture (European Finches Prestige, Versele-Laga), tap water and sand grit with crushed shell oyster.

Birds' status was checked daily to ensure their welfare and health. The veterinary also checked for birds' general state and housing conditions. In July, after the experiments, the birds were transferred to a large room (2.72 × 1.55 × 2.20 m) for flight training for 7 days. Individuals were then released at their respective capture sites where they joined groups of conspecifics.

We measured birds' body mass (with a scale), tarsus and wing lengths (with a calliper). Ectoparasite mite load on wing feathers was assessed by an estimating method following Behnke et al. (1999; 1995). We performed a principal component analysis (PCA) on untransformed morphological measurements (body mass, tarsus and wing lengths), for the twenty females to use the score of the principal component (PC) as a measure of body size. The PCA revealed one PC, characterized for positive loadings for the three variables, explaining 49% of total variation (trait loadings: body mass 0.561, tarsus 0.792 and wing lengths 0.720).

Colouration measurements

Females are less colourful than males, being almost colourless in the forehead and exhibiting a yellow patch in the breast which is less well defined and interspersed by some brownish-grey lines along it (Cramp and Perrins 1994). The female's yellow patch was measured with an Ocean Optics USB4000 spectrophotometer (Dunedin, FL, USA), with a deuterium and halogen light source (Mikropack Mini-DT-2-GS, UV-VIS-NIR), emitting light covering the 300nm to 700nm interval, and an optical fibre reflectance probe (Ocean Optics R400-7 UV/VIS), held vertically, attached to a rigid black holder to standardize the distance between probe and sample (3 mm), providing a sampling area of 28 mm^2. All measurements of the spectrum were expressed in the proportion of light relative to a white standard (Ocean Optics, WS-1-SS White Standard). We took three readings for each of the sampled areas: throat, breast and belly. We then averaged the three areas for data reduction. We calculated saturation according to Leitão et al. (2014), from spectral reflectance data between 320 and 700nm, extending to the UV region, to which birds are sensitive (Cuthill 2006). We used saturation to discriminate individuals as this is positively correlated with carotenoid content of the feathers (Butler et al. 2011; Saks et al. 2003) and is the chosen signal in males is carotenoid saturation (Leitão et al. 2014).

Male mate choice

The first experiment was conducted between April and May 2014. Based on saturation, females were allocated to a more colourful group or a drabber group (more colourful group n= 10; less colourful group n= 10). Pairs of females were formed selecting one from each group to maximize the differences between ranked females. The pairs of females had no differences in body size (ANOVA $F_{1, 19} = 1.442$ $P = 0.245$) or parasite load ($F_{1, 19} = 0.062$ $P = 0.807$). Males (n= 20) were presented with a unique

pair of females. The mate choice experiments were performed from 8h to 12h a.m., with birds exhibiting the species typical breeding behaviour in their cages. The mate choice experiments were performed in a T shaped test room with three compartments: a larger one (155 x 272 X 220 cm) connected to two smaller ones (112 x 136 x 220 cm;(See Figure 1 of Leitão et al. 2014). The closest area to the glass was considered as the choice area and time spent by males in the interaction area of females was used as a measure of male preference (Nolan and Hill 2004 and wherein references). The females were randomly assigned to each of the two smaller compartments and the male was placed in the larger compartment. Females could see the male but not each other, and all birds had vocal contact. The trials lasted 60 minutes, being the first 30 minutes considered acclimatization period. Each trial was video-recorded for subsequent analysis with Observer XT 10 software (Noldus, Wageningen, The Netherlands). We performed 20 tests, but three were discarded due to lack of interaction between birds. We measured the time spent in the choice area and number of hops in the perches.

Social competition

We conducted a second experiment, in June 2012, using 16 of the females that were tested in the mate choice experiment, to test for intrasexual competition. A context of competition for access to a limited food source in new formed groups of females was created. Initially 4 groups, each constituted of 4 randomly chosen females that had not been housed together when in captivity, were formed. The groups were formed 2 days before the experiment trials, housed in cages similar to the housing cages with water and food in ad libitum. Individuals were coloured-ringed (green, light blue, dark blue and red), for visual identification. The experiment consisted in filming social interactions between individuals in a feeding context. In each trial the animals were deprived of food for 2 hours (12h00) and then one feeder

was placed inside and in the middle of the cage (14h00), which would allow for just only one animal to feed at a time. Trials lasted 15 minutes. Each group was subjected to to 5 trials that took place in consecutive days. This experiment never caused physical injury.

Video tests were analysed by performing focal observations of each individual, using the software Observer XT 10 (Noldus Information Technology). Intrasexual competition was analysed through aggressiveness and monopolisation of the feeder. We registered the number of attacks and displays made and the identity of the receivers of this behaviours, being attacks a physical confront towards the opponent and display being threatening another bird with head down and open beak, with or without rapid wings movement. ”Aggressiveness” was calculated as the sum of number of attacks and displays made by each individual. A dominance score was calculated for each individual in each group using the data of the 5 days. Taking into account that the interactions were dyadic we used normalized David’s score (David 1987) for each bird, with the correction for different interaction frequencies between individuals (de Vries et al. 2006). David’s score quantifies an individual’s overall success by considering wining interactions weighted by its opponents’ wins and losses in the group (David 1987). Higher scores are indicative of higher dominance. After calculating the dominance score we examined the dominance relationship between members of each group calculating the steepness of the dominance hierarchy (de Vries et al. 2006). Steepness measures the range of individual differences in dyadic interactions (values close to 1 indicate strong dominance hierarchies whether values close to 0 indicate a shallow hierarchy (de Vries et al. 2006)). We also analysed the activity of each individual as the total number of hops in the perches and in the feeder. We had to interrupt one of the group experiments since one female became sick, so that the analysis was restricted to 3 groups of 4 females.

Statistical procedures

In order to understand the signal content of plumage saturation of females, we performed a Generalised Linear Model (GLM), with saturation as a dependent variable. Female age entered as factor, body size and parasite load entered as covariates in the model. For the analysis of male preference we built a GLM with repeated measures, with male association time as the dependent variable. We used a Linear Model defining the number of the trial as the subject for pairwise comparison. Female age entered as factor, female saturation and the time females spent in the response area of male, number of female hops and body size entered as covariates in the model.

In the social dominance experiment we first tested for group differences in aggressiveness, mean time spent in the feeder and activity, with an ANOVA with the average score for each individual as the dependent variable and group as a random factor. We estimated within-individual repeatability of aggressiveness, mean time spent in the feeder and activity, as well as changes in these across the 5 days from a ANOVA (Lessells and Boag 1987). We then examined the relationship between aggressiveness, dominance score, mean time spent in the feeder and activity. We used a GLMM to test if dominance score (average score per individual across the five days) were related with body size, age (first year old versus older), parasites or saturation. Group was used as a random factor in order to control for possible differences in aggressiveness between groups. Statistical analyses were performed with software IBM SPSS Statistics ® 20.0 for Windows.

Ethical note

This study was conducted in accordance with legal Portuguese agencies Instituto de Conservação da Natureza e Florestas and Direcção Geral de Veterinária, and under ICNF permits 48 / 2012 / CAPT for animal capture and maintenance.

RESULTS

None of the factors considered – age, body size and parasite load - were significant predictors for saturation of female plumage (Table 1).

Table 1 General linear model for female saturation, with age, body size and parasite load as independent variables. N = 20; values of the estimates and standard error (SE); Wald χ^2 test and probability (P).

Explanatory Variables	Estimates ± SE	Wald χ^2	*P*
(Intercept)	0.957 ± 0.037	902.506	0.000
Age	-0.051 ± 0.035	2.16	0.142
Body size	0.008 ± 0.020	0.172	0.678
Parasite load	-0.002 ± 0.012	0.041	0.840

Male mate choice

In the mate choice trails, males were clearly motivated having spent 83.4 ± 22.5% of trial time in the response area of females. However, males did not show any preference

for female colouration (Table 2). Instead, time males spent in the response area was associated with time females spent in their response areas, so males seemed to respond to female presence. Other factors as females' age or physical condition did not significantly affect the time males' spent on their choice area (Table 2).

Table 2 Generalized linear model, with repeated measures, for male preference in mate choice trials. N = 17; values of the estimates and standard error (SE); Wald χ^2 test and probability (P).

Explanatory Variables	Estimates ± SE	Wald χ^2	*P*
(Intercept)	695.897 ± 805.769	0.696	0.404
Saturation	-426.311 ± 818.984	0.271	0.603
Time in response area	0.393 ± 0.120	10.720	**0.001**
Number of hops	-2.541 ± 1.527	2.769	0.096
Age	-68.577 ± 137.215	0.250	0.617
Body size	-82.715 ± 68.364	1.464	0.226

Social competition

The groups did not differ in social behaviour (aggressiveness: $F_{9,11}$= 0.289, p= 0.756; mean time spent in the feeder: $F_{9,11}$= 1.066, p= 0.384) and general activity ($F_{9,11}$= 0.309, p= 0.742). The within-individual repeatability in aggressiveness was significant across days (r= 0.480; $F_{11,44}$= 5.624, P< 0.001), as the time that individuals spent

monopolizing the feeder (r=0.276; $F_{11,44}$= 2.909, P= 0.006). In the same way, moderate within-individual repeatability was found in activity (r= 0.184; $F_{11,44}$= 2.134, P= 0.037).

Aggressiveness was strongly related with the time individuals spent monopolizing the feeder (rs= 0.592, df= 12, P= 0.043) but not with activity (rs= 0.91, df= 12, P= 0.778). Dominance score was also highly related with aggressiveness (rs= 0.757, df= 12, P= 0.004), and with time in the feeder (rs= 0.692, df= 12, P=0.013) but not with activity (rs = -0.417, df= 12, P= 0.178). Individuals higher in dominance were more aggressive and were also the ones that controlled the food container for longer. The steepness of dominance relationships was high (X= 0.776 ± 0.123, Figure 1), which is indicative of strong hierarchies. Although more aggressive individual are socially dominant, we did not find any characteristic either physical (saturation and body size), of condition (parasite load) or age, that was indicative of female status (Table 3).

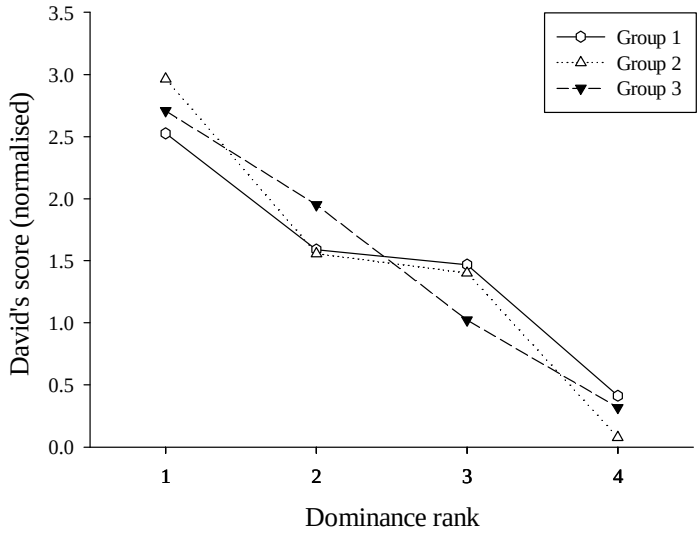

Figure 1 Dominance hierarchy steepness. Relation between David's score (normalised) and the rank of each individual within each group (3 groups). The absolute value of the slope for each group measures the steepness of the hierarchy. Each symbol represents different individuals within-groups.

Table 3 Generalized linear mixed model for the social dominance score, group number was used as random factor. N = 12; values of the estimates and standard error (SE); F test and probability (P).

Explanatory Variable	Estimates ± SE	F	P
Intercept			
Saturation	0.889 ± 17.273	0.003	0.961
Age	3.670 ± 4.245	0.747	0.427
Body size	-1.080 ± 2.428	0.198	0.675
Parasites	0.553 ± 1.775	0.097	0.768

DISCUSSION

Through a series of experiments, we found that carotenoid-based plumage of female serins was not sexually discriminated by males and was not related to social dominance over food. Our results suggest that, in the European serin female colouration seems to be a result of genetic correlation, as it is not used in mate choice neither in social competition for food.

Male mate choice

In the mate choice experiments, we found that when males had to choose between a colourful and a drabber female, they did not exert a choice. Instead, the most relevant aspect was that male association time was clearly associated with female association

time. That is, males preferred females that spent more time closer to them. Apparently, males were just being responsive to the interest of females rather than choosing between them. These results do not support our first and second hypotheses and support the idea that female colouration is not a sexual signal. Theory assumes that in species with conventional sex roles, males do not discriminate instead they try to fertilize as many females as possible (Jones et al. 2002; Trivers 1972). However, in species with high male reproductive investment, males may become choosier, and there can be variance of investment with male condition: scorpion flies' males in poor condition invest more when mating with high-quality females (Engqvist and Sauer 2001). In monogamous species with high bi-parental care, mutual mate choice is expected occur, because female quality could have a great impact in male reproductive success (Amundsen and Pärn 2006).

From field observations it is known that male serins are keen on courting every female they find if not mate-guarded, and extra-pair copulation attempts are extremely common, albeit rarely successful (Mota and Hoi-Leitner 2003). It agrees with this behaviour in natural settings the fact that in our experiment males were more responsive to females that they were exerting any choice. Males simply approached and courted the females that revealed interest in them, which could either be for pair-bonding or for extra-pair behaviour.

In previous studies the possibility for male mate choice in birds yield mixed results. Amundsen and co-workers (1997) found that males associated more with more colourful females of *Luscinia s. svecica*. In *Parus caeruleus*, males preferred UV reflecting females over no UV reflecting females (Hunt et al. 1999). Hill (1993) showed that *Carpodacus mexicanus* males preferred brighter females, although female colouration was not related to condition, reproductive success or winter survival. If female colouration is not an indicator of individual quality, male selection for brighter females may have evolved as a correlated response (Hill 1993). On other cases such as the slightly dimorphic *Hirundo rustica* Cuervo and colleagues (1996) were unable to find mutual mate choice based on tail length. Also, in *Agelaius phoeniceus*, the red

epaulets displayed by females revealed no function in sexual selection or dominance interactions and the authors concluded that this trait evolved as a correlated response to selection for male colouration (Muma and Weatherhead 1989).

The serin is a sexually dichromatic species with a yellow patch that is selected on males by females (Leitão et al. 2014). It has been correlated with survival (Figuerola and Senar 2007) and condition during moult (Figuerola et al. 2003). This patch is carotenoid-dependent, which may be a signal indicator of good foraging ability, since carotenoids have to be incorporated in the diet. The same reasoning applies to females. And if males are choosy on their prospective mates, they should pay attention to traits that could indicate their foraging ability, as the yellow patch. This means that sexual selection on ornaments by males should occur particularly when the ornament is related to female quality. However, we were unable to find a correlation between plumage colouration and female quality in this species. We just considered some measures of female condition and we cannot dismiss that female coloration may be related with other quality traits.

There are several possible explanations for the absence of choice by males in a species with high paternal investment. One possibility is that males have no advantage in being choosy. The cost of selecting a pair may be higher than not choosing at all. There is a risk of remaining unmated; the time and energy of sampling could be too high for males; the cost of predation could increase while sampling for a mate; nest or young predation could be higher in more conspicuous mates (reviewed in Jennions and Petrie 1997). For example, female plumage colouration is negatively correlated with nest predation in wood warblers (*Parulinae*) and finches (*Carduelinae*) (Martin and Badyaev 1996). Another possible explanation is that male choice could be less obvious (Engqvist and Sauer 2001), and males simply invest less in low-quality females (Roulin 1999). This differential investment is a less extreme strategy (Cunningham and Birkhead 1998) and it is possible that different forms of selection operate on the ornaments of sexes (Heinsohn et al. 2005; LeBas 2006). Selection criteria in both sexes could be different and males may select females based on other traits (Hill 1993).

Another explanation is that ornamentation is costly, and if there is a trade-off between ornamentation and reproduction, males should not evolve a preference for more colourful females, which could have reduced fecundity (Fitzpatrick et al. 1995) or offspring with lower survival (Clutton-Brock 2007). Females invest directly in their offspring instead of in ornaments.

Social competition

The social competition experiment showed that female yellow colouration does not function as a status signalling during competition for access over food. Although experimental groups formed steep hierarchies, i.e. there were strong difference between individuals in their dominance rank, we could not find any signal mediating female disputes. Using the same experimental protocol used in the present study for social competition, we found that in male serins yellow carotenoid-based colouration is a reliable predictor of social status (Leitão et al unpublished), while in this study female colouration did not relate to social dominance. Therefore, we show that the same trait may function differently in females and males.

This intra-specific variation between signal function in females and males could be due distinct life history strategies while foraging. Previous work with this species in the field has shown that while foraging, male serins mainly directed aggressions towards females (Senar and Domènech 2011), suggesting that males are dominant over females. As serins colouration is carotenoid-based and dependent of diet, this signal could be an indicator of good foraging ability that is linked to food monopolisation which males are more efficient than females.

Research made in the function of traits as status signalling in both sexes is still limited, as most of it was performed only in males. Some studies have described this comparison in monochromatic species: in the American goldfinch *Spinus tristis*, female carotenoid-based bill functions as a signal of status in contests over food (Murphy et al.

2009b), while in males it doesn't (Murphy et al. 2014). In the rock sparrow *Petronia petronia*, male patch size was correlated with access to food (Griggio et al. 2007), and the same was also true in females (Griggio et al. 2010). Although female serins present a reduced signal by comparison with males, it was admissible that the trait has similar functions in both sexes, which is not the case. More studies are needed to understand if status signalling can function on both males and females.

In general terms, colouration may function in status signalling in social interactions over mating resources or other nonsexual resources (besides food), like territories (West-Eberhard 1983; Amundsen 2000, LeBas 2006; Kraaijeveld et al. 2007). Here, we tested this signalling function over access to food and we did not find any effect of colouration in mediating the disputes. However, we cannot exclude that female yellow colouration may function in other contexts beyond access to food. Future studies should take into account other contexts to assess if female ornaments do function in social status signalling.

Conclusion

In summary our results show that female plumage colouration is not a signal in mate choice and in social competition over food. Also, we did not find a correlation between individual quality (physical condition or parasite load) and plumage colouration in females. While these results are based on lack of evidence, previous results using the same experimental design both in mate choice (Leitão et al 2014, Trigo et al unpublished) and in social competition (Leitão et al unpublished), have shown effects of male yellow colouration in female mate choice and in male competition over food. Thus, this reinforces the idea that coloration in female serins is not involved in social or sexual signalling.

Taken together, these results indicate that female coloration in this species is not sexually or socially selected as a sexual or social signal. Most likely it is the result of

genetic correlation over male selected coloration. Thus, female ornaments seem to be by-products of sexual selection on males' ornaments

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CHAPTER 6

Conclusions

This study provided information about the honesty, message and mechanism of carotenoid-based plumage ornamentation in males and females of the European serin *Serinus serinus*.

In chapter 2, I show that the availability of a non-pigmentary carotenoid, β-carotene, affected the total plasma carotenoids concentration. Besides, it enhances the response to a cellular immunity challenge and saturation of the plumage colouration. Finally, in chapter 2 it is also demonstrated that this trait is sexually selected. The main conclusions of this chapter are that yellow carotenoid-based colouration may be an indirect indicator of general condition, which can also be dependent on carotenoids. This result is in accordance with new theories revisiting the mechanism by which carotenoids modulate colouration (Hartley and Kennedy 2004). Traditionally, it was supposed that there was a trade-off for carotenoids between colouration and health (Lozano 1994), with several supporting evidences (Blount et al. 2003; McGraw and Ardia 2003). Recently, some authors arguments that an alternative to this hypothesis is that colouration is an indirect indicator of general health, linked to the biochemical efficiency of vital cellular processes (Hill and Johnson 2012).

In chapter 3, I wanted to explore the message of the colour signal in a field work by relating colouration with several parameters. With a four year study, with 100 birds, I found that plumage colouration of males serins could be predicted by age and ectoparasite load. Older birds had bigger yellow patches and with higher values of plumage chromaticity; birds less parasitized had plumages with bigger patches and higher values of achromaticity, however had lower values of plumage chromaticity. These results suggest that this sexual selected trait may signal direct and indirect benefits for females. Females may benefit of older and experienced males and with less risk of parasitic infection and offspring may benefit of higher resistance to parasites.

Since there are fundamental differences between avian and human vision (Cuthill 2006), and in order to accurately quantify what is perceived by birds, special measuring

techniques are required. In this work I compared different colour metrics: tristimulus and tetrachromatic visual models. I found that tristimulus colour variables and avian visual models were highly correlated. With these results I established that carotenoid-based plumage colouration is a condition-dependent trait (Simons et al. 2012; Romero-Díaz et al. 2013) and is predicted by age (Evans and Sheldon 2012; Laucht and Dale 2012; Grunst et al. 2014) and parasites (Biard et al. 2010; Martínez-Padilla et al. 2010; Mougeot et al. 2009).

Many male secondary sexual traits are regulated by androgens (Hau 2007). In birds, androgens modulate the expression of melanin-based colouration (Bókony et al. 2008; Muck and Goymann 2011), and some carotenoid-based colouration of bare parts and skin. However, the androgen-dependence of carotenoid plumage colour is not yet well established. I investigated the role of androgens in the development of the expression of plumage colours in serins during moult (chapter 4). I found a negative effect of plasma testosterone levels on the size of the yellow ornament. I also found that testosterone treatment negatively affected the UV-chroma and saturation. Neither plasma testosterone levels nor testosterone treatment affected other colouration variables neither physical condition, revealing a limited effect of this androgen in colouration and general condition. These results are surprising since it was expected that testosterone modulate males ornamentation in some manner. These results do not agree with both the hypothesis proposed to explain the testosterone regulation of secondary sexual traits: the immunocompetence handicap hypothesis (ICHH) (Folstad and Karter 1992) and the oxidation handicap hypothesis (OHH) (Alonso-Alvarez et al. 2007). Accordingly, examples of carotenoid-based plumage colouration modulated by androgens are scarce and generally linked to species with pre-nuptial moult (Lindsay et al 2009; Lindsay et al 2011) or to beaks, bare parts and skins (Casagrande et al. 2011; Pham et al. 2014).

In chapter 5 I explored the function of the yellow ornamentation in the plumage of female serins. Female ornamentation can function as an adaptive and non-adaptive

signal: sexual or social function or genetic correlates of selection on males. A few studies had shown the possible dual function of female ornamentation (Kraaijeveld 2004, Griggio 2010). However, I did not find a sexual function for plumage ornamentation, instead males prefer females that interacted more with them. I couldn't also find a social function of this trait in serin females, as female formed steep dominance hierarchies in a food context; it was not related to colouration. These results point to a genetic correlation of the males' trait on carotenoid-based plumage colouration of female European serins.

The main conclusions of this thesis were that carotenoid-based plumage colouration in males European serin is a sexually selected condition-dependent trait. This trait is an honest signal, giving information about carotenoid plasma levels, immune responses, age, and ability to cope with parasite infection. However, I found no evidence that plumage colouration was androgen-dependent. I also explored the function and message of carotenoid-based plumage colouration in females, and I found that the signal in females is not condition-dependent, nor sexually selected, nor social selected. I concluded that the expression of the signal in females is a genetic correlation with males' trait.

A further direction of work should be on the function of the signal as a status signal in males and study of the mechanisms leading to feather pigmentation. The specific mechanisms leading the dietary carotenoids to carotenoid pools and to the utilization in different physiological functions, including colouration, immunity, antioxidation, should be investigated. Furthermore, a research about the reproductive success of males and females related with colouration could disclosure some unanswered questions.

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